Hypolipidemic, Antioxidative and Vascular Effects of Soy Leaves (*Glycine max* L. Merr.)

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Abstract

Cardiovascular disease is one of major causes leading to death in the world. Previous studies suggest that soybean may be beneficial in preventing cardiovascular disease. It was proposed that genistein and its glycosides, which are present in soybean, might be responsible for this health benefit. However, up to now, there have been no studies about the chemical composition and health benefits of soy leaves even though soy leaves are abundantly available.

In the present study, the chemical composition and some health benefits of soy leaves were investigated. By using high performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR), six different kaempferol glycosides were identified and isolated from soy leaves. They are named as kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside, kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside, kaempferol-3-O-digalactopyranoside, kaempferol-3-O-diglucopyranoside, kaempferol-3-O-α-L-rharmnopyranosyl(1→6)-β-D-galactopyranoside and kaempferol-3-O-rutinoside. The amount of kaempferol glycosides present in soy leaves was 22.4 mg/g. It was shown that soy leaves also contained genistein and its glycosides, but the amount was lower than that in soybean (0.54 mg/g in soy leaves and 2.12 mg/g in soybean).

The effect of soy leaves on the ratio of non-high-density lipoprotein cholesterol (non-HDL-C) to high-density lipoprotein cholesterol (HDL-C) was studied in hamsters fed a high fat and high cholesterol diet. Supplementation of 3% soy leaves powder (SLP) or 3% soy leaves ethanol extract (SLEE) in their diet could significantly lower this ratio to 0.79 ± 0.11 in the SLP group ($p < 0.01$), 0.78 ± 0.18 in the SLEE group ($p < 0.05$) compared to that of the control (0.92 ± 0.08).

In the antioxidation experiments, Cu^{2+}-mediated LDL oxidation and inhibition of erythrocyte haemolysis were used to assess the antioxidative ability of soy leaves and its kaempferol glycosides. Results indicated that soy leaves butanol extract possessed a weak antioxidative ability against the Cu^{2+}-mediated LDL oxidation and all the six kaempferol glycosides showed no inhibition in this assay. However, significant inhibitions on erythrocyte haemolysis was observed in the soy leaves butanol extract and all the kaempferol glycosides were inhibitory in a dose-dependent manner.

In the blood vessel relaxing experiments, soy leaves butanol extract relaxed artery rings pre-constricted by 9,11-dideoxy-11_{α},9_{α}-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619) or [5Z,9_{α},11_{α},13E,15S]-9,11,15-trihydroxyprosta-5,13-dienoic acid ($PGF_{2\alpha}$) in a dose-dependent manner. None of the kaempferol glycosides affected vessel tension induced by U46619. Results suggested that an active vasorelaxant other than

the kaempferol glycosides were presented in soy leaves.

In the last part of this study, female Sprague-Dawley rats were used as an animal model to determine the effect of soy leaves on tumor induction. Supplementation of 3% soy leaves powder in the diet could neither decrease the incidence rate nor the number of tumors induced by dimethylbenz[a]anthracene (DMBA).

In conclusion, soy leaves may possess some beneficial effects in humans through the actions of modifying the lipoprotein profile to lower risk of cardiovascular disease, scavenging of the free radicals to decrease the oxidative stress and dilating the arteries to decrease the risks caused by hypertension.

摘要

部份研究指出大豆是有利於防治心臟血管的疾病。可是到目前爲止，仍沒有研究是關於大豆葉的化學成份或其對人體之益處。

在本研究中，我們發現大豆葉含有六種不同之山奈酚糖甙，並且成功地把它們在大豆葉中分離出來。

在利用蒼鼠作爲動物模型進行大豆葉對血脂影響的實驗中，把 3% 的大豆葉粉末或 3% 的大豆葉乙醇提取物加進高脂和高膽固醇的飼料中來餵飼它們。實驗結果發現在該兩組的蒼鼠，他們的非高密度脂蛋白和高密度脂蛋白的比例較對照組的一組爲低。

在測試大豆葉抗氧化能力的實驗中，大豆葉的丁醇提取物對銅離子誘導的低密度脂蛋白氧化有一定程度的抑制作用。我們亦使用了紅血球溶解的實驗來測試大豆葉的丁醇提取物及分離出來的六種山奈酚糖甙的抗氧化能力，結果顯示它們對紅血球的溶解均擁有隨劑量而定的抑制作用。

在血管放鬆的實驗之中，大豆葉的丁醇提取物能有效地以隨劑量而定的方式來放鬆預先利用 9,11-dideoxy-11$_{\alpha}$,9$_{\alpha}$-epoxy-methanoprostaglandin F$_{2\alpha}$ (U46619) 或 [5Z,9$_{\alpha}$,11$_{\alpha}$,13E,15S]-9,11,15-trihydroxyprosta-5,13-dienoic acid (PGF$_{2\alpha}$) 收縮的頸動脈。實驗結果顯示在大豆葉內含有可放鬆血管的物質。

在本研究的最後一部份，我們利用了雌性的 S.D.大白鼠來作爲實驗模型去研究有關大豆葉對乳腺癌的影響。實驗結果顯示把 3% 的大豆葉粉末加進它們

的飼料之中來餵飼它們並不能有效地降低由 dimethylbenz[a]anthracene (DMBA) 所誘發的乳腺癌的病發率或其之總數。

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Chapter 1
General introduction

1.1 History of soybean

The rich history of the soybean (*Glycine max* L. Merr.) began over five thousand years ago on the windy plains of eastern Asia. According to the Chinese tradition, soybeans were one of the five sacred crops named by Chinese emperor Sheng-Nung. Historians maintained that Sheng-Nung mentioned soybeans in his *Ben Tsao Gang Mu*, written in the year 2838 B.C. By 300 B.C., soybeans and millet were always mentioned in ancient texts as the two major food crops in northern China.

Once the soybean was cultivated by Chinese farmers, it spread gradually from the northern part to the southern part of China. It was then spread into Korea, Japan, Southeast Asia and Europe.

Soybeans yield more usable protein per acre than does any other crop and far more inexpensive protein than do animal foods. In China, with its shortage of pasture and arable farm land, nutrient-rich soybeans are an ideal food. Also, the Buddhist commitment to vegetarianism has led monks to create soy-based meat substitutes.

1.2 Health benefits of soybean

Recently, the health effects of soybean have been extensively investigated. The growing evidence has shown that soybean may play important roles in the prevention of certain cancers (Kennedy 1998; Lamartiniere *et al.* 1998; Thiagarajan *et al.* 1998; Wei *et al.* 1998; Wu *et al.* 1998; Pollard and Wolter 2000), reduction of the risk of osteoporosis (Alekel *et al.* 1998; Arjmandi *et al.* 1998 and Williams *et al.* 1998) and heart disease (Huff *et al.* 1982; Wilcox *et al.* 1995; Anthony *et al.* 1996; Balmir *et al.* 1996; Anthony *et al.* 1998; Nilausen and Meinertz 1998; Palacio *et al.* 1998; Potter *et al.* 1998a and 1998b; Wilson *et al.* 1998; Wong *et al.* 1998; Anderson *et al.* 1999; Ho *et al.* 2000 and Merz-Demlow *et al.* 2000) . These health benefits of soybean are believed due to isoflavones, which are abundant in soybean seeds.

1.3 Introduction to flavonoids

Flavonoids are polyphenolic compounds that are ubiquitously present in foods of plant origin. Flavonoids are categorised into flavonols, flavones, flavanones, catechins and isoflavones. All of them are structurally-related to the parent compound, flavone (2-phenylbenzopyrone) (Figure 1.1).

1.4 Bioavailability of flavonoids from foods

The absorption and subsequent distribution, metabolism and excretion of flavonoids in humans have not been extensively studied. Absorption of flavonoids from the diet was long considered to be negligible, as most flavonoids are present in foods bound to sugar as β-glycosides. Only free flavonoids without a sugar molecule, the so called aglycon, were considered to be able to pass the gut wall because no enzymes that can split these predominantly β-glycosidic bonds are secreted into the gut or present in the intestinal wall (Hollman *et al.* 1995). However, micro-organisms in colon are indispensable for hydrolysis of the β-glycosidic bonds (Kuhnau 1976 and Hollman and Katan 1999). Hollman *et al.* (1995) showed that absorption of orally administered quercetin aglycon was 24%, whereas the absorption of quercetin glycosides from onions was much higher (52%), and the absorption of pure quercetin rutinoside was 17%. This study showed that humans absorb appreciable amounts of quercetin and that absorption of glycosides in the small intestine is possible. Andlauer *et al.* (2000) showed that genistin, the glycoside of genistein, was partly absorbed without previous cleavage of the β-glycosidic bond. Other studies showed similar results that the glycosides of quercetin had been found in human plasma (Paganga and Rice-Evans 1997). These studies indicate that flavonoid glycosides may be able to enter the human serum and

exert their effect directly. It has been estimated that humans consume approximately one gram of mixed flavonoids per day (Pierpoint 1986).

1.5 Pharmacological effects of flavonoids and their glycosides

In recent years, the flavonoids and their glycosides have attracted much attention in relation to their physiological and biological properties, such as a anticarcinogenic, antioxidative, cardioprotective, osteoprotective, neuroprotective and antiangiogenic.

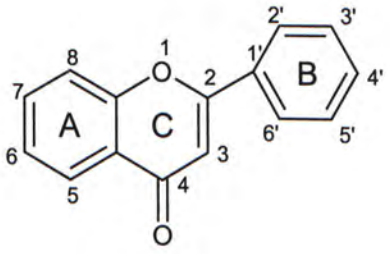
1.5.1 Anticarcinogenic activity

Some studies have examined the anticarcinogenic activity of flavonoids (Peterson and Barnes 1996; Wei *et al.* 1998; Denis *et al.* 1999;Griffiths *et al.* 1999; Dimas *et al.* 2000; Mukhtar and Ahmad 2000; Steele *et al.* 2000). Isoflavones, a kind of flavonoids which are rich in soybean, are dietary components of plant origin with weak estrogenic activity (Kuiper *et al.* 1998). Epidemiologic data indicate that higher intakes of soy protein are associated with a lower rate of breast cancer (Lee *et al.* 1991; Messina *et al.* 1994; Wu *et al.* 1998). These epidemiologic data were supported by the observations that genistein decreased development of breast tumors in experimental animals and slowed the growth of human breast cancer cells in vitro

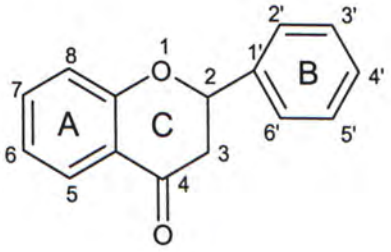
(Barnes 1995a). *In vitro* studies showed that genistein inhibited both estrogen and growth factor simulated proliferation of human breast cancer lines MCF-7, T47D ER^+ and T47D ER^- in culture with IC_{50} values ranging from 7.0 to 9.4 μM/mL (Peterson and Barnes 1996). Breast cancer growth is regulated by estrogen and peptide growth factors, such as epidermal growth factor (EGF), the receptor of which has intrinsic protein tyrosine kinase (PTK) activity. Akiyama *et al.* (1987) showed that genistein could act as a specific inhibitor of PTK. Therefore, genistein may block mammary epithelial cell growth by interfering with signal transduction events stimulated by estradiol or growth factors. Setchell *et al.* (1984) suggested that genistein inhibited tumor cell growth by an antiestrogenic mechanism through competition with E_2 for occupancy of the estrogen receptor (ER).

Genistein was also shown to inhibit 7, 12-dimethylbenz[a]anthracene (DMBA) induced and 12-O-tetradecanoyl phorbol-13-acetate (TPA) promoted skin carcinogenesis in mice (Wei *et al.* 1998).

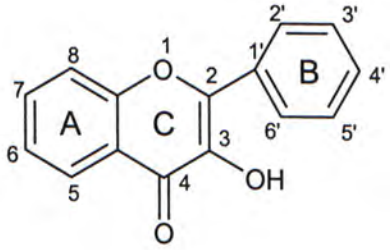
Dimas *et al.* (2000) showed that kaempferol glycosides exhibited a cytotoxic activity against human leukaemic cell lines CCRF-CEM, H33AJ-JA13, HUT 78, H9 (T cells), NAMALWA, JIYOYE, CCRF-SB, K562 and U937 with IC_{50} values ranging from 6.5 to 30.7 mg/mL.



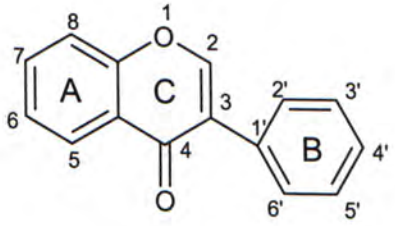
Flavone



Flavanone



Flavonol



Isoflavone

Figure 1.1 Chemical structures of some naturally occurring flavonoids.

	Substituent(s) at position:							
Flavonoid	5	6	7	8	2'	3'	4'	5'
Flavones								
Apigenin	OH		OH				OH	
Chrysin	OH		OH					
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3		OCH_3	OCH_3	
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3			OCH_3	OCH_3
Flavonols								
Kaempferol	OH		OH				OH	
Quercetin	OH		OH			OH	OH	
Galangin	OH		OH					
Fisetin			OH			OH	OH	
Myricetin	OH		OH			OH	OH	OH
Morin	OH		OH		OH		OH	
Flavanones								
Naringenin	OH		OH			OH		
Hesperetin	OH		OH			OH	OCH_3	
Isoflavones								
Biochanin A	OH		OH			OCH_3		
Genistein	OH		OH			OH		
Daidzein			OH			OH		

1.5.2 Antioxidative activity

Free radicals can induce oxidation of compounds such as lipids, proteins and even DNA single-strand breakage. The damage to biosystem is one of the major processes that contributes to degenerative diseases such as cancer, cardiovascular disease and aging.

Antioxidant is a compound capable of inhibiting oxygen-mediated oxidation of diverse substrates, from simple molecules to polymers and complex biosystem. There are two types of antioxidants. The first type inhibits formation of free radicals, which may initiate oxidation. In most cases they are chelators of metal ions. The second type inhibits free-radicals chain-propagation reactions.

Flavonoid is a kind of natural antioxidants which exhibit antioxidative and free radical scavenging activities. Studies showed that flavonoids can inhibit LDL oxidation and may be able to attenuate atherosclerosis (Aviram and Fuhrman 1998; Kerry and Abbey 1998; Zhu *et al.* 1999, 2000).

Apart from antioxidative ability of flavonoids in LDL, Asgary *et al.* (1999) showed that kaempferol could inhibit non-enzymatic glycosylation of amino group of lysine residue in haemoglobin which is usually caused by high glucose concentration in diabetes. These haemoglobin glycosylation is an oxidative reaction, therefore, antioxidant can be able to prevent this damage on haemoglobin.

And Noroozi *et al.* (1998) demonstrated that flavonoids are also effective in preventing DNA damage on human lymphocytes. The effective dose that would result in a 50% reduction in oxidative damage (ED50) for kaempferol, quercetin and vitamin C were 104 μmol/L, 40 μmol/L and 233 mmol/L from 100 μmol H_2O_2 /L respectively. They showed that flavonoids exhibited a higher antioxidative effect that vitamin C. Mitchell *et al.* (1998) showed that the antioxidative capacity of kaempferol and quercetin were higher than vitamin C as determined by trolox equivalent antioxidant capacity (TEAC) and ferric reducing ability of plasma (FRAP) assay.

The antioxidative activity of flavonoids may be related to the hydorxyl groups in the phenolic rings to produce a phenoxyl radical (Figure 1.2). The reduction potential of these groups are influenced by the number and their positions on the molecule. Noroozi *et al.* (1998) showed that the aglycons quercetin, kaempferol, luteolin and myricetin had a greater antioxidative capacity than do the conjugate flavonoids. The high reductive capacity of kaempferol in ESR and FRAP assay may due to the presence of the 4'-OH on the B-ring and the 3-OH on the C-ring (Figure 1.1) which are connected via the π-orbital system and interact synergistically.

The antioxidative activities of these flavonoids may change as a result of solvent effects and pH which can influence the state of protonation or deprotonation

of the hydroxyl groups. Therefore, the antioxidative activity of flavonoids may differ significantly in different model systems depending on the reduction potentials of the hydroxyl moieties relative to that of the oxidizing radical, or transition metal ion, used in the system (Mitchell *et al.* 1998).

In the microsomal system, another important factor that determines the antioxidative efficiency of these flavonoids is their ability to partition between aqueous and lipid phases.

1.5.3 Cardioprotective activity

Epidemiological data suggest that dietary flavonoid intake is inversely associated with mortality from coronary heart disease (Hertog *et al.* 1993; Arai *et al.* 2000; Ho *et al.* 2000).

Apart from the antioxidative effect of flavonoids that can decrease the chance of atherosclerosis, flavonoids may also be beneficial in improving plasma lipid profile. Soy isoflavone decreased non-high density lipoprotein cholesterol (non-HDL-C) and increased HDL-C (Anthony *et al.* 1996, 1998; Merz-Demlow *et al.* 2000). The effect of lowering low-density lipoprotein cholesterol (LDL-C) and increasing HDL-C by isoflavones may be due to their weak estrogenic effect. These isoflavones can bind to estrogen receptors (Kuiper *et al.* 1998) and share the LDL reducing and HDL

increasing effects of human estrogen (Lilley *et al.* 1998; Westerveld 1998; Godsland 2001). The mechanism of how estrogen reduces serum LDL and increase HDL is still unclear but it is proposed that estrogen induces the LDL-receptor activity and increases the rate of LDL elimination in the liver.

1.5.4 Osteoprotective activity

Arjmandi *et al.* (1998a, 1998b) showed soy isoflavones can significantly increase the bone density of ovarian hormone-deficient rats. They suggested that the serum alkaline phosphatase activity (ALP), an index of bone formation , tended to be higher in soy-fed animals, indicating a positive effect on bone formation.

Another proposed mechanism is that genistein can act as an inhibitor of tyrosine kinases which can directly modulate osteoclastic acid sectretion (Akiyama *et al.* 1987). This acid secreted onto the bone matrix can dissolve the bone mineral. This process is under complex control related to the maintenance of serum calcium activity and bone strength, disorders of which may cause osteoporosis. Thus, substances influencing bone resorption may be a potential antiosteoporotic agents (Williams *et al.* 1998).

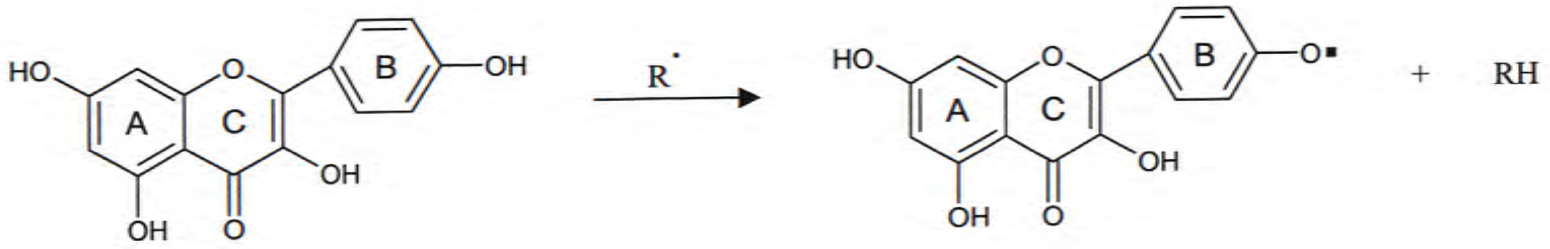


Figure 1.2 Free radical ($R^{\cdot}$) scavenge by flavonoids

1.5.5 Neuroprotective activity

Alzheimer's disease (AD) is one of the major neurodegenerative diseases. Amyloid β protein (Aβ), the major protein component of senile plaque, can elicits a toxic effect on neurons. It has been suggested to play an important role in pathogenesis of AD. Wang *et al.* (2001) showed that 50 μM kaempferol and apigenin decreased the Aβ-induced cell death by 81.5% and 49.2% respectively. However, quercetin and luteolin failed to protect neurons. They suggested that the substitution of hydroxyl group at C-3' position severely impaired the neuroprotective ability of kaempferol.

1.5.6 Antiangiogenic activity

Angiogenesis, the generation of new capillaries, is virtually absent in the healthy adult organism and is restricted to a few conditions including wound healing and the formation of corpus luteum, endometrium, and placenta. However, if angiogenesis is not tightly regulated, it is obligatory for the growth and progression of solid cancers. Fotsis *et al.* (1993) showed that genistein was a potent inhibitor of endothelial cell proliferation and *in vitro* angiogenesis at concentrations giving half-maximal inhibition of 5 and 150μM, respectively. Another studies showed that flavonoid may inhibit angiogenesis through the inhibition on the secretion of a

primary angiogenic cytokine, vascular endothelial growth factor (VEGF), by human prostate and breast cancer epithelial cells (Jiang *et al.* 2000). Therefore, flavonoids may inhibit the growth and progression of solid cancers through the inhibitory effect of angiogenesis.

1.6 Soybean leaves

To my best knowledge, there was no study about the health effects of soy leaves and its chemical composition. Soy leaves not only contain isoflavones but also contain some valuable flavonoid glycosides (details will be discussed in Chapter 2). Therefore, the health benefits of soy leaves deserve further investigation. In the present study, the biological activities of soy leaves and its different flavonoid glycosides were examined.

Chapter 2

Isolation and purification of kaempferol glycosides and genistin in soy leaves

2.1 Introduction

Flavonoids and their glycosides are polyphenolic compounds which are widely distributed in fruits, vegetables and nuts. Naturally occurring flavonoids are classified as flavones, flavonols, flavanones, isoflavones and catechins. They are structurally-related to the parent compound, flavone (2-phenylbenopyrone). It has been estimated that humans consume approximately 1g of mixed flavonoids per day (Pierpoint 1986).

Flavonoids have many beneficial effects including being antioxidative (Arora *et al.* 1998; Kerry and Abbey 1998; Mitchell *et al.* 1998; Noroozi *et al.* 1998 and Zhu *et al.* 2000), anti-carcinogenic (Akiyama *et al.* 1987; Fotsis *et al.* 1993 and Peterson and Barnes 1996), anti-atherosclerosis (Anthony *et al.* 1998), and relaxative to blood vessel (Mishra *et al.* 2000; Figtree *et al.* 2000). It is well known that soybean contains a large amount of isoflavones, however, up to date, there is no study that has been done on the isolation and purification of similar compounds from the leaves of soybean.

2.2 Objectives

The present study was to isolate and purify the known and unknown flavonoids and their glycosides from soy leaves (*Glycine max* L. Merr). Their antioxidative and relaxing effects in rat carotid artery rings were then examined.

2.3 Materials and Methods

2.3.1 Extraction and isolation

2.3.1.1 Preparation of soy leaves butanol extract

Dried soy leaves (3 kg) were extracted with 18 L 70% ethanol three times at 50 °C. Ethanol was evaporated under vacuum to yield 1550 g ethanol extract, which was then dissolved in 2 L distilled water. Then the ethanol extract was partitioned with chloroform in a ratio of 1:1 for three times. The chloroform fraction was dried down under vacuum to yield 98 g chloroform extract. The remaining ethanol extract was further partitioned with butanol in a ratio of 1:1 for three times. The butanol and water fractions were dried down under vacuum to yield 110 g butanol extract and 1280 g water extract.

2.3.1.2 Preparation of kaempferol glycosides from soy leaves butanol extract

In brief, 105 g soy leaves butanol extract was fractionated in a column packed with Sephadex LH-20 (100 μM, Pharmacia Fine Chemical Co., Ltd., Germany) and eluted with 4.4 L absolute methanol (400 mL each time) to give rise to eleven fractions (S1 to S11). Fractions S1, S2, S3 and S11 contained mainly sugars, organic acids and some other components with no UV absorption, while S4 - S10 contained mainly a mixture of glycosides. The total weight of the fractions S4 to S10

was 81 g. Each fraction of S4- S10 was then loaded onto a Sephadex LH-20 column. For S4, S5 and S8, pure methanol was used, while for S6, S7, S9 and S10, 40% acetone was used to elute the column. The elutes were monitored using a HPLC equipped with a UV detector. These fractions containing flavonoids or glycosides were then loaded onto a C18 HPLC preparative column (Hypersil ODS, 250 x 22mm, 10 μm, Alltech, Deerfield, IL, USA) and eluted with water and acetonitrile, leading to purification of six compounds. The chemical structures and the molecular weights of these glycosides were identified using their ultraviolet (UV), infrared (IR), mass, ^{1}H-NMR and ^{13}C-NMR spectrum. The purity of these flavonoid glycosides and genistin were greater than 95%, based on HPLC analysis.

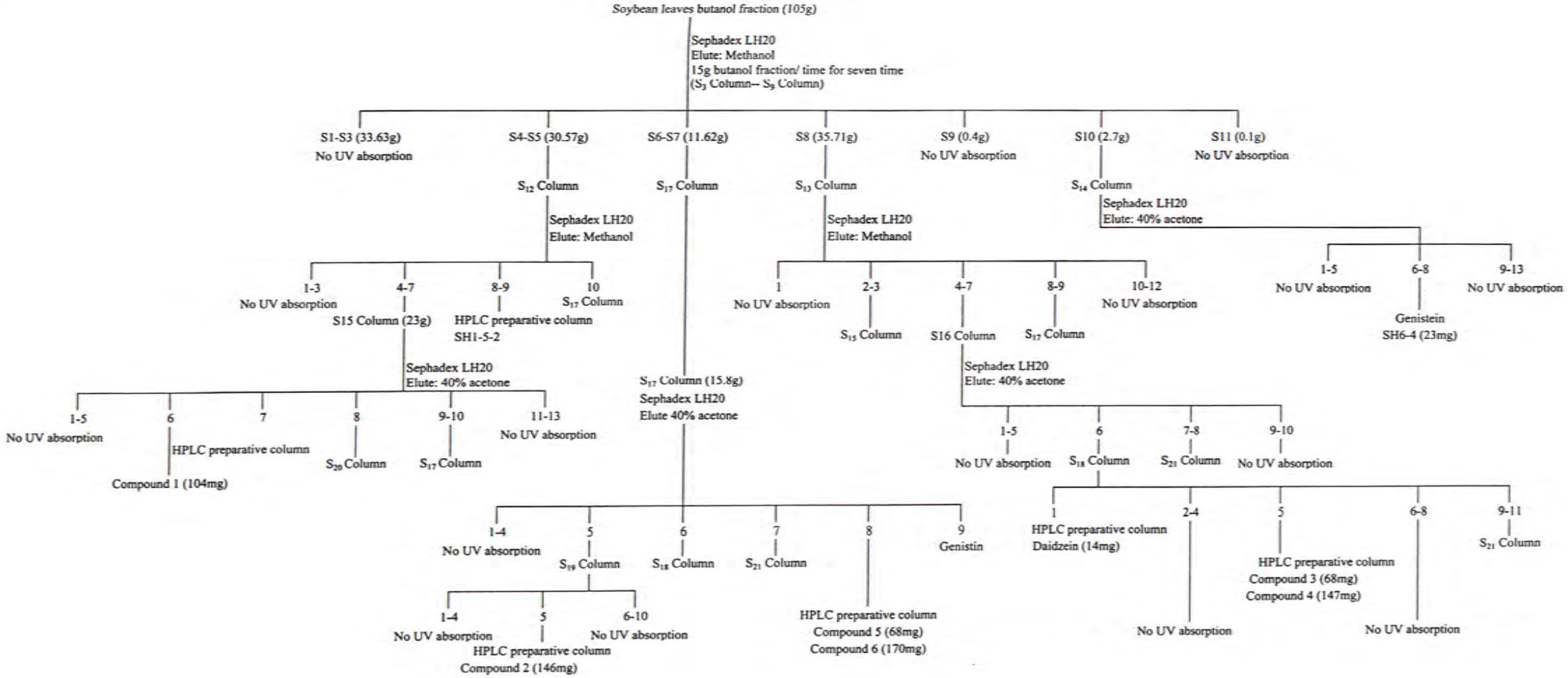


Figure 2.1 Flow chart for the isolation of the flavonoids and their glycosides from soy leaves (*Glycine max* L. Merr).

2.3.2 High performance liquid chromatography (HPLC) analysis

2.3.2.1 Sample preparation for the HPLC analysis

All wet samples were freeze-dried before the HPLC analysis. One gram of dried, finely ground samples was placed in a 100 mL conical flask containing 40mL methanol, 20 mL 0.1 N HCl and 0.2 mL flavone (11.25 mM in methanol). The mixture was sonicated for 10 minutes and then stirred at room temperature for 2 hours using a magnetic stirrer. Extractants were centrifuged at 850 g for 10 minutes. The supernatant of sample was filtered through a 0.45 μm PTEE filter [poly(tetrafluoroethylene), Alltech Associates Inc., Deerfield, IL]. The supernatant was transferred into a 1.5 mL sample vial (Hewlett Packard, Palo Alto, CA, USA) and then subjected to HPLC analysis.

2.3.2.2 HPLC analysis

All the flavonoids and their glycosides were analyzed using a Hewlett Packard series 1100 HPLC (Hewlett Packard, palo alto, CA, USA) equipped with a binary pump delivery system (G1322A) and a diode array detector (G1946A).

In brief, 10 μL of the supernatant was loaded onto a C18 column (Hypersil ODS, 4.6 x 250 mm, 5 μM, Waters, Ireland) through an autosampler (G1313A). The diode array detector was set from 200 to 400 nm, and the eluting components were

monitored at 260 nm. The quantitative analysis of the flavonoids and their glycosides were carried out according to the method of Wang and Murphy (1994) with some modifications. The mobile phase consisted of 1% acetic acid in water (v/v) (solvent A) and acetonitrile (solvent B). After injection of the sample, solvent B was increased from 10 to 20% in 40 min and then increased from 20 to 100% in the next 30 min. The flow rate was maintained at 0.8 mL/min. Two typical HPLC chromatograms of soybean and soy leaves were shown in Figures 2.2 and 2.3 respectively.

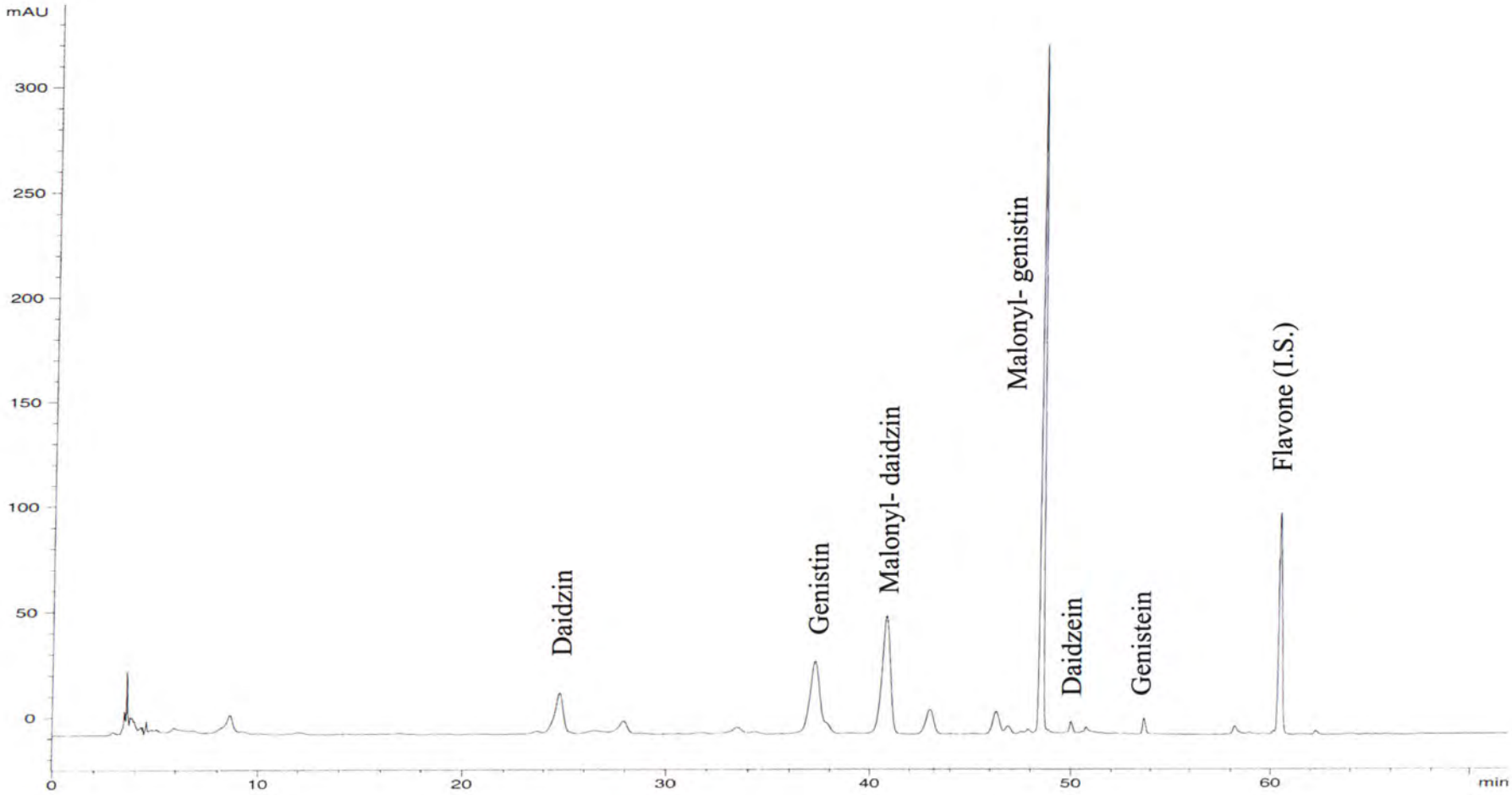


Figure 2.2 HPLC chromatogram of soybean seed extract

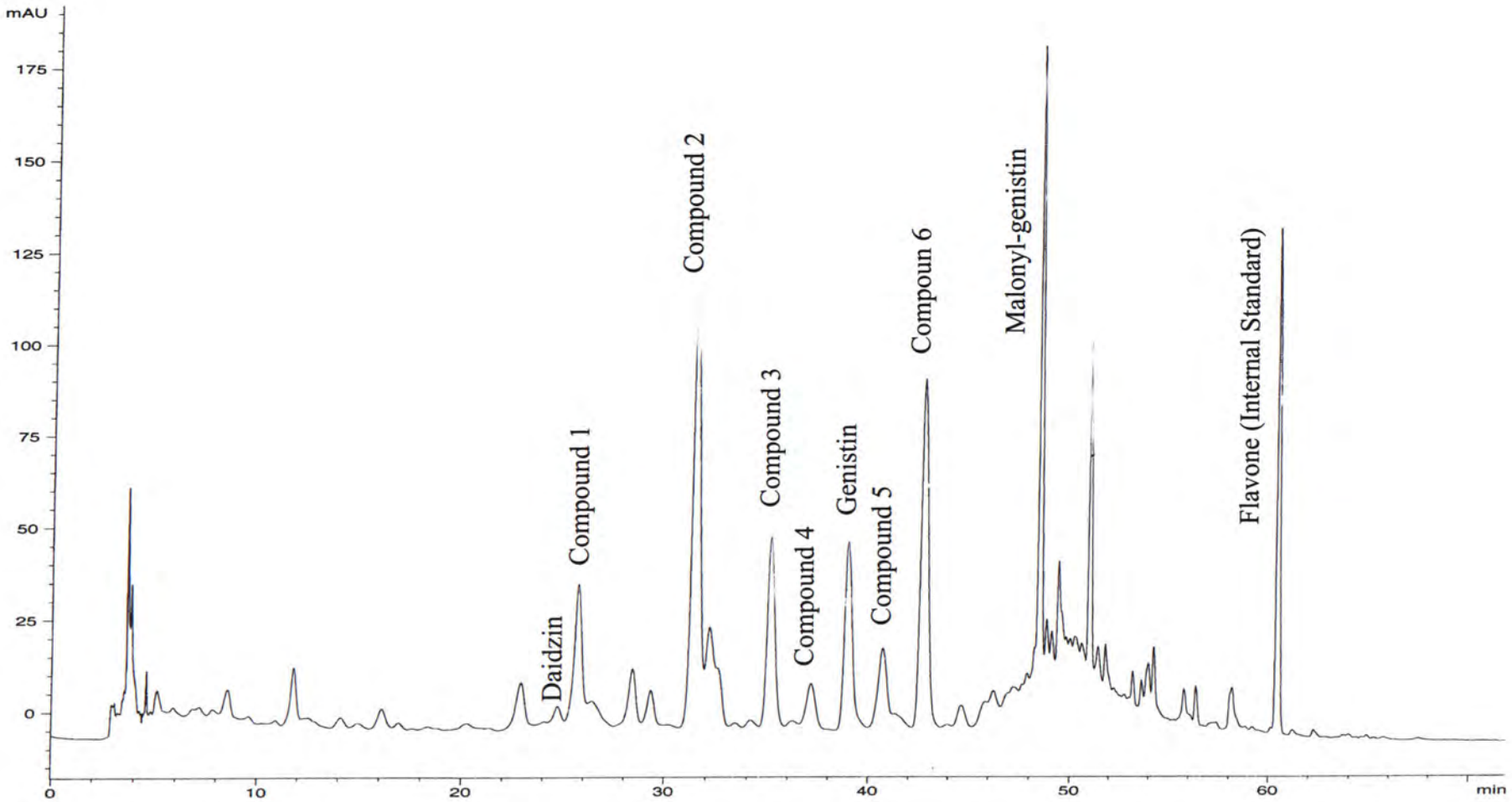


Figure 2.3 HPLC chromatogram of soy leaves extract

2.3.2.3 Quantification of the flavonoids and their glycosides

The flavonoids and their glycosides were quantified by comparing area responses with those of authentic standards or the compounds purified by the method as described in part ***2.3.1.2***. Authentic standards for genistein, daidzein and kaempferol with a purity higher than 98% were obtained from Sigma Chemical Company (St. Louis, MO, USA). The concentrations of malonyl and acetyl glycosides of genistein and daidzein were calculated from the curves for corresponding β-glycosides, corrected for molecular weight differences since the molar extinction coefficient of the esterified isoflavone is similar to that of the β-glycosides (Barnes *et al.* 1994).

2.3.2.4 Change in flavonoids and their glycosides in soy leaves

Soy leaves were planted in Meng County in Henan in China in the period from June to September. Three different batches of soy leaves samples were collected at the end of each month. The flavonoids and their glycosides were determined by the method described in part ***2.3.2.1- 2.3.2.3***.

2.4 Results

The structures of six unknown compounds were identified by studying their various spectra of UV, IR, Mass, ^{1}H and ^{13}C-NMR. The characteristics of each compound were described below and the structures were illustrated in Figures 2.4 and 2.5.

2.4.1 Compound 1

Yellow amorphous powder, m.p. 199-201 °C, $[\alpha]_D^{20}$–71.87 (c0.064, MeOH), $C_{33}H_{40}O_{20}$. *m/z*: 757 $[M+1]^+$, 611 [M-rhamnosyl+1]$^+$, 499[M-rhamnosyl-glucosyl+1]$^+$ and 287 [M-rhamnosyl-glycosyl-galactosyl+1]$^+$; UV λ_{Max}^{MeOH} nm: 266, 300sh, 348; IR υ_{Max}^{KBr} cm^{-1}: 3408, 1658, 1608, 1508, 1361, 1207, 1178, 1072, 889, 893, 814; ^{1}H and ^{13}C NMR.

2.4.2 Compound 2

Yellow amorphous powder, m.p. 203-205 °C, $[\alpha]_D^{20}$ -87.8 (C0.660, MeOH), $C_{33}H_{40}O_{19}$. *m/z*: 741 $[M+1]^+$, 595 [M-rhamnosyl+1]$^+$, 499 [M- 2x rhamnosyl+1]$^+$, 287 [M- 2x rhamnosyl-galactosyl+1]$^+$; UV λ_{Max}^{MeOH} nm: 266, 300sh, 349; IR υ_{Max}^{KBr} cm^{-1}: 3361, 1660, 1610, 1508, 1450, 1360, 1209, 1178, 1136, 1051, 978, 837, 812; ^{1}H and ^{13}C NMR.

2.4.3 Compound 3

Yellow amorphous powder, m.p. 196-198 °C, $[\alpha]_D^{20}$ –23.7 (c0.050, MeOH), $C_{27}H_{30}O_{16}$. *m/z*: 611 $[M+1]^+$, 449 [M-galactosyl+1]$^+$, 287 [M- 2x galactosyl+1]$^+$, UV λ_{Max}^{MeOH} nm: 267, 300sh, 352; IR υ_{Max}^{KBr} cm^{-1}: 3330, 1660, 1606, 1498, 1361, 1279, 1257, 1207, 1078, 1018, 889, 841; ^{1}H and ^{13}C NMR.

2.4.4 Compound 4

Yellow amorphous powder, m.p. 198-200 °C, $[\alpha]_D^{20}$ –23.7 (c0.050, MeOH), $C_{27}H_{30}O_{16}$. *m/z*: 611 $[M+1]^+$, 449 [M-glucosyl+1]$^+$, 287 [M- 2x glucosyl+1]$^+$; UV λ_{Max}^{MeOH} nm: 266, 315sh, 351; IR υ_{Max}^{KBr} cm^{-1}: 3377, 1657, 1606, 1504, 1361, 1281, 1211, 1178, 1080, 1030, 887, 841; ^{1}H and ^{13}C NMR.

2.4.5 Compound 5

Yellow amorphous powder, m.p. 228-230 °C, $[\alpha]_D^{20}$ –17.28 (c0.045, MeOH), $C_{27}H_{30}O_{15}$. *m/z*: 595 $[M+1]^+$; UV λ_{Max}^{MeOH} nm: 265, 300sh, 350; IR υ_{Max}^{KBr} cm^{-1}: 3410, 1655, 1606, 1500, 1360, 1209, 1178, 1084, 1057, 841, 841; ^{1}H and ^{13}C NMR.

2.4.6 Compound 6

Yellow needle shaped crystal, m.p. 182-185 °C, $C_{27}H_{30}O_{15}$. *m/z* 595 $[M+1]^+$; UV λ^{MeOH}_{Max} nm: 266, 310sh, 350; IR υ^{KBr}_{Max} cm^{-1}: 3408, 1655, 1606, 1506, 1450, 1360, 1211, 1178, 1065, 1014, 885, 839, 808; ^1H and ^{13}C NMR.

Table 2.1 UV absorptions of compound 1-6 (λ_{max}) in different solutions

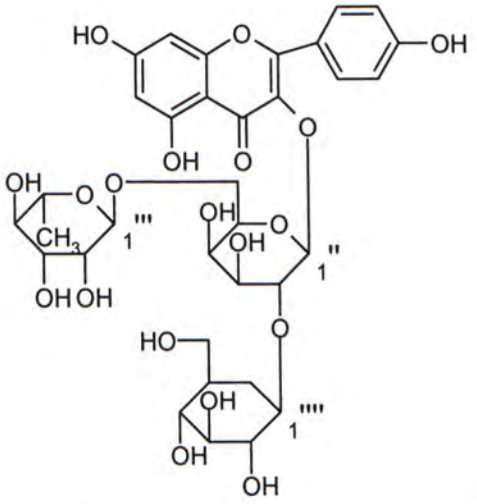
	MeOH	NaOMe	$AlCl_3$	$AlCl_3$ / HCl	NaOAc	NaOAc/ H_3BO_3
Compound 1	266 300sh 348	274 323 393	274 305 352 399	276 305 348 399	273 308 382	263 300sh 353
Compound 2	266 300sh 349	274 323 393	275 303 347 397	275 305 350 399	267 301 365	267 301sh 348
Compound 3	267 300sh 352	275 326 402	274 304 351 398	275 304 347 398	275 312 392	268 296 351
Compound 4	266 315sh 351	275 326 402	272 305 350 399	275 303 346 399	274 309 389	266 300sh 355
Compound 5	265 300sh 350	275 326 402	274 305 351 397	275 303 351 397	274 305 383	266 298 351
Compound 6	266 310sh 350	275 326 402	274 303 352 398	274 304 350 397	274 315 394	266 310sh 353

Table 2.2 ^{1}H NMR spectra of compound 1-6 in DMSO-d_6

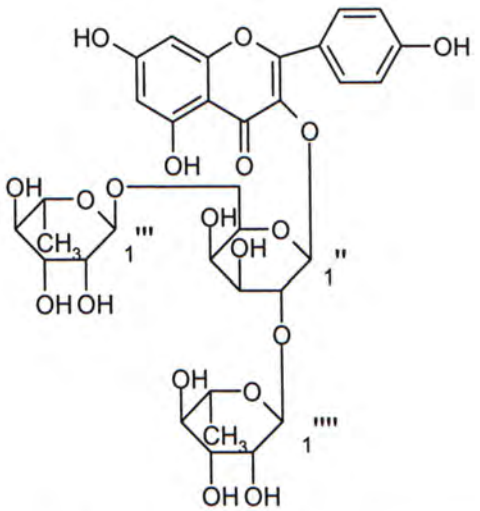
	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	*Kaempferol*
3-OH	-	-	-	-	-	-	9.37
5-OH	12.60	12.68	-	12.58	-	12.55	12.47
6	6.18(s)	6.16(s)	6.17(d1.5)	6.18(d2.1)	6.11(s)	6.20(d2.1)	6.18(d3.0)
7-OH	-	-	-	10.20	-	10.83	10.77
8	6.40(s)	6.38(s)	6.39(d1.5)	6.40(d1.8)	6.32(s)	6.40(d2.1)	6.43(d3.0)
2′ 6′	8.05(d)	8.03(d9.0)	8.06(d7.0)	8.03(d9.0)	8.03(d9.0)	8.00(d9.6)	8.03(d15)
3′ 5′	6.84(d)	6.85(d8.0)	6.86(d7.0)	6.89(d9.0)	6.85(d9.0)	6.87(d9.6)	6.91(d15)
4′-OH	-	-	-	10.20	-	10.10	10.10
1″	5.56(d8.0)	5.55(d8.0)	5.34(d7.5)	5.36(d7.2)	5.27(d7.5)	5.32(d7.0)	-
2″-6″	2.81-3.80(m)	3.32-4.36(m)	2.91-4.06(m)	2.80-3.38(m)	3.01-3.62(m)	3.15-3.69(m)	-
1‴	5.04(s)	5.04(s)	3.77(d6.5)	4.04(d7.5)	4.4(s)	5.07(d8.0)	-
2‴-5‴	3.33-3.71(m)	3.10-3.74(m)	2.84-3.95(m)	2.80-3.38(m)	3.09-3.62(m)	3.42-4.08(m)	-
6‴	0.77(d6.0)	1.04(3H d6.0)	3.71(d3.0) 3.38(d3.0)	-	1.05(d6.3)	0.98(d6.3)	-
1⁗	4.02(d8.0)	5.04(s)	-	-	-	-	-
2⁗-6⁗	3.12-3.77(m)	-	-	-	-	-	-
6⁗	3.33(m) 2.96(d8.5)	0.78(3H d6.0)	-	-	-	-	-

Table 2.3 ^{13}C NMR spectra data of compound 1-6 in DMSO

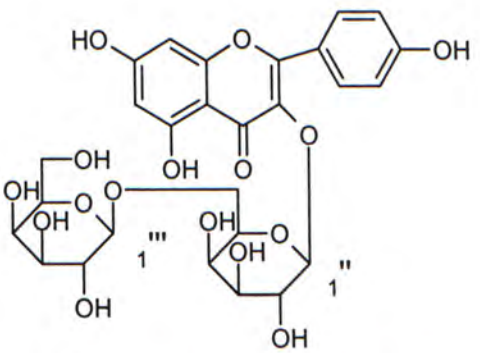
	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	kaempferol
2	156.20	156.35	156.34	156.29	156.42	156.26	146.60
3	132.71	132.67	133.20	133.05	133.01	133.0	135.48
4	177.29	177.30	177.32	177.11	176.78	177.12	175.56
5	161.19	161.24	161.16	160.95	160.84	160.97	160.48
6	98.89	98.67	98.97	98.64	99.93	98.60	98.08
7	164.46	164.01	165.15	164.07	166.55	163.87	163.64
8	93.76	93.68	93.85	93.66	93.93	93.63	93.37
9	156.38	156.35	156.50	156.29	155.96	156.62	155.95
10	103.90	103.98	103.63	103.02	102.86	103.86	102.92
1′	120.90	120.91	120.84	120.72	120.66	120.73	121.51
2′	130.83	130.82	130.94	130.79	130.71	130.71	129.34
3′	115.07	115.07	115.08	114.96	114.92	114.94	115.29
4′	159.90	159.85	159.08	159.70	159.82	159.66	158.96
5′	115.07	115.09	115.08	114.96	114.92	114.94	115.29
6′	130.83	130.82	130.94	130.79	130.71	130.71	129.34
1″	102.90	98.97	102.89	103.87	102.21	101.20	
2″	74.80	74.83	71.11	74.03	71.83	74.10	
3″	73.62	73.80	73.75	76.18	73.39	76.27	
4″	68.43	68.53	69.87	69.66	68.21	69.86	
5″	73.23	73.33	76.59	76.49	72.94	75.66	
6″	67.08	65.14	67.15	67.94	65.24	66.82	
1‴	100.58	100.08	103.63	100.92	99.25	100.65	
2‴	70.68	70.42	72.93	73.34	70.34	70.28	
3‴	70.59	70.58	73.27	76.49	70.55	70.52	
4‴	71.84	71.88	67.81	69.66	71.05	71.70	
5‴	68.18	68.18	76.59	76.49	67.94	68.19	
6‴	17.26	17.92	60.88	60.74	17.96	17.76	
1⁗	98.70	100.58					
2⁗	73.76	70.42					
3⁗	76.58	70.58					
4⁗	69.87	71.88					
5⁗	76.58	68.28					
6⁗	60.87	17.26					



Compound 1. kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside

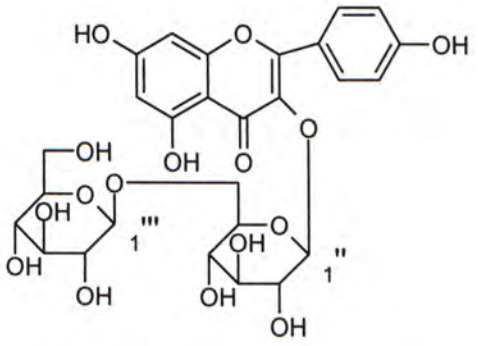


Compound 2. kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside

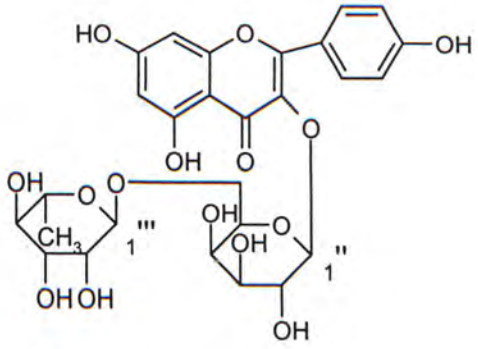


Compound 3. kaempferol-3-O-digalactopyranoside

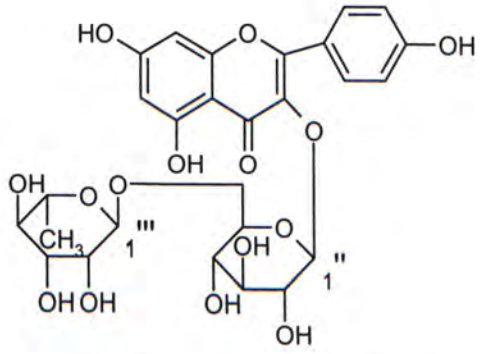
Figure 2.4 Structures of compounds 1-3



Compound 4. kaempferol-3-O-diglucopyranoside



Compound 5. kaempferol-3-O-α-L-rharmnopyranosyl(1→6)-β-D-galactopyranoside



Compound 6. kaempferol-3-O-rutinoside

Figure 2.5 Structures of compounds 4-6

2.4.7 Quantification of flavonoids in soybean and soy leaves

The flavonoids in soybean and in soy leaves were quantified from the standard curves of their authentic standards or pure compounds (purified in part 2.3.1.2) with flavone as an internal standard (Table 2.4).

2.4.8 Age-dependent changes in flavonoids and their glycosides

The three different batches of soy leaves samples were collected from Meng county, Henan, China. Their flavonoids and their flavonoid glycosides in these soy leaves were determined and shown in Figure 2.6. It was found that total flavonoids and their glycosides reached highest at month 3.

Table 2.4 The compositions of flavonoids in soybean and in soy leaves (mg/g)

	Soybean	Soy leaves
Daidzin	0.32 ± 0.02	ND
Genistin	0.54 ± 0.03	0.09 ± 0.02
Malonyl-daidzin	0.98 ± 0.19	ND
Malonyl-genisin	1.56 ± 0.01	0.31 ± 0.01
Daidzein	0.02 ± 0.01	ND
Genistein	0.02 ± 0.01	ND
Compound 1	ND	0.32 ± 0.01
Compound 2	ND	7.06 ± 0.10
Compound 3	ND	1.08 ± 0.06
Compound 4	ND	0.88 ± 0.03
Compound 5	ND	5.04 ± 0.14
Compound 6	ND	8.02 ± 0.16
Total	3.45 ± 0.27	22.80 ± 0.29

Data are expressed as mean ± S.D. (n=3)

ND: Not detected

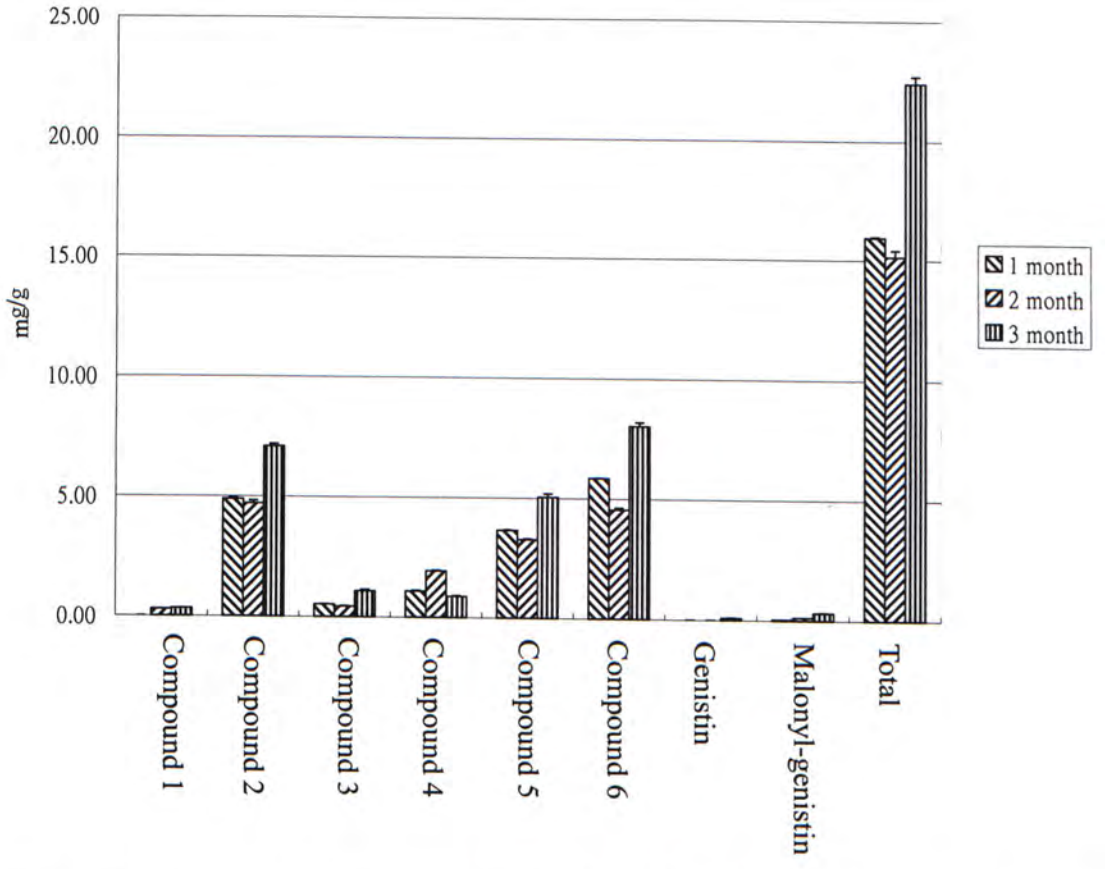


Figure 2.6 Change in flavonoids and their glycosides in soy leaves over the time of growth

2.5 Discussion

2.5.1 Compound 1

Compound 1, was isolated as a yellow amorphous powder, m.p. 199-201 °C, $[\alpha]_D^{20}$ –71.87 (c0.064, MeOH), with molecular formula $C_{33}H_{40}O_{20}$ deduced from ^1H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^+$ peak at *m/z* 757 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated the formula weight was 756. The APCI-MS spectrum of compound 1 revealed peaks at *m/z* 611 [M-rhamnosyl+1]$^+$, *m/z* 499[M-rhamnosyl-glucosyl+1]$^+$ and *m/z* 287 [M-rhamnosyl-glycosyl-galactosyl+1]$^+$, indicating compound 1 was a glycoside containing 3 different sugars. The ^1H NMR signals due to the aglycon at δ 6.18 ppm (1H, s), δ 6.40 ppm (1H, s), δ 8.05 ppm (2H, d, J= 8.5 Hz), δ 6.84 ppm (2H, d, J= 9 Hz), showed the characteristic pattern of kaempferol derivatives. From the ^{13}C NMR spectrum, the downfield shift of C-3 by 2.7 ppm, indicated the bonding site of the sugar group was at C-3 position (Markham *et al.* 1978). The HMBC (Heteronuclear Mutiple Bond Coherence) spectrum also showed important correlation between the H-1 [δ 5.58 ppm] of the galactose and C-3 [δ 132.7 ppm] of the aglycon. This further proved that the glycosylation site was at C-3 position of the aglycon. The data of the UV spectrum (Table 2.1) indicated the presence of hydroxyl groups at C-7 and C-5 positions (Hillis and Isoi, 1965). Acid

hydrolysis of compound 1 led to form a aglycon kaempferol. The sugars remained after acid hydrolysis were proved to be rhamnose, galactose and glucose by TLC (Thin Layer Chromatography) with their standards.

From the ^{13}C NMR spectrum, the C-2 of the galactose showed a downfield shift by 2 ppm, indicated the rhamnose was linked at C-2 position of the galactose. The C-6 of the galactose at δ 67.88 ppm performed a downfield shift by 6.2 ppm and the C-6 of the glucose at δ 60.87 ppm showed a (6→1) linkage between the galactose and the glucose.

Compound 1 was deduced as kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside (Figure 2.4).

2.5.2 Compound 2

Compound 2, was isolated as a yellow amorphous powder, m.p. 203-205 °C, $[\alpha]_D^{20}$-87.8 (C0.660, MeOH), with a formula $C_{33}H_{40}O_{19}$ deduced from ^1H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^+$ peak at *m/z* 741 in the APCI-MS, indicated the formula weight was 740. The APCI-MS spectrum of compound 2 revealed peaks at *m/z* 595 $[M\text{-rhamnosyl}+1]^+$, *m/z* 499 [M- 2x rhamnosyl+1]$^+$ and *m/z* 287 [M- 2x rhamnosyl-galactosyl+1]$^+$. Acid hydrolysis of compound 2 led to form a aglycon kaempferol. The sugars were proved to be

rhamnoses and glucose by TLC with their standards. The results of the UV absorption spectrum, ^{1}H NMR and ^{13}C NMR were compared with the data from Fico *et al.* (2000), proving that compound 2 was kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside (Figure 2.4).

2.5.3 Compound 3

Compound 3, was isolated as a yellow amorphous powder, m.p. 196-198 °C, $[\alpha]^{20}_{D}$ –23.7 (c0.050, MeOH), with molecular formula $C_{27}H_{30}O_{16}$ deduced from ^{1}H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^{+}$ peak at *m/z* 611 in the APCI-MS, indicated the molecular weight was 610. The APCI-MS spectrum of compound 3 revealed peaks at *m/z* 449 $[M\text{-galactosyl}+1]^{+}$ and *m/z* 287 $[M\text{- 2x galactosyl}+1]^{+}$. The data of the UV spectrum (Table 2.1) showed the presence of hydroxyl groups at C-5 and C-7 positions (Hillis and Isoi, 1965). From the ^{13}C NMR spectrum, the upfield shift of C-3 by 2.2 ppm, indicated the glycosylation site was at C-3 position (Markham *et al.* 1978). The C-6 of the galactose showed a downfield shift by 6.3 ppm, showed a (1→6) linkage between the two galactoses. Acid hydrolysis of compound 3 led to form a aglycon kaempferol. The sugars after acid hydrolysis were proved to be galactose by TLC with the standard galactose. Compound 3 was deduced as kaempferol-3-O-

digalactopyranoside (Figure 2.4).

2.5.4 Compound 4

Compound 4, was isolated as a yellow amorphous powder, m.p. 198-200 °C, $[\alpha]_D^{20}$ –23.7 (c0.050, MeOH), with molecular formula $C_{27}H_{30}O_{16}$ deduced from ^1H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^+$ peak at *m/z* 611 in the APCI-MS, indicated the molecular weight was 610. The APCI-MS spectrum of compound 4 revealed peaks at *m/z* 449 $[M\text{-glucosyl}+1]^+$ and *m/z* 287 $[M-\text{ 2x glucosyl}+1]^+$. Compound 4 showed similar values of UV spectrum with compound 3, indicated that compound 4 has a similar structure with compound 3. From the ^{13}C NMR spectrum of compound 4, the C-6''' of the glucose showed a downfield shift by 6.2 ppm, indicated a (1→6) linkage between the two glucose molecules. After acid hydrolysis of compound 4, a aglycon kaempferol was formed. The sugars after acid hydrolysis were proved to be glucose by TLC with the standard glucose. Compound 4 was deduced as kaempferol-3-O-diglucopyranoside (Figure 2.5).

2.5.5 Compound 5

Compound 5, was isolated as a yellow amorphous powder, m.p. 228-230 °C, $[\alpha]_D^{20}$ −17.28 (c0.045, MeOH), with molecular formula $C_{27}H_{30}O_{15}$ deduced from ^1H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^+$ peak at *m/z* 595 in the APCI-MS, indicated the molecular weight was 594. The ^1H NMR signals due to the aglycon at δ 6.11 ppm (1H, s), δ 6.32 ppm (1H, s), δ 8.03 ppm (2H, d, J= 9.0 Hz) and δ 6.85 ppm (2H, d, J= 9.0 Hz), showed the characteristic pattern of kaempferol derivatives. ^1H NMR also showed two anomeric protons signals at δ 5.27 ppm (1H, d, J= 7.5 Hz) and δ 4.4 ppm (1H, s). Furthermore, the ^{13}C NMR showed a upfield shift of C-3 by 2.4 ppm, these data indicated the presence of two sugar moieties in compound 5 and the bonding site of the sugar group was at the C-3 position (Markham *et al.* 1978). The ^1H NMR spectrum gave signals δ 1.05 ppm (3H, d, J= 6.3 Hz) indicated one of the sugar was rhamnose. ^{13}C NMR showed another sugar was galactose. Also the ^{13}C NMR showed the C-6 of the galactose shifted downfield by 5 ppm, indicated a (1→6) linkage between the rhamnose and the galactose. Acid hydrolysis of compound 5 yielded a aglycon kaempferol. The sugars after acid hydrolysis were proved to be rhamnose and galactose by TLC with their standards. Compound 5 was deduced as kaempferol-3-O-α-L-rhamnopyranosyl (1→6)-β-D-galactopyranoside (Figure 2.5).

2.5.6 Compound 6

Compound 6, was isolated as a yellow needle shaped crystal, m.p. 182-185 °C, with molecular formula $C_{27}H_{30}O_{15}$ deduced from ^{1}H NMR (Table 2.2), ^{13}C NMR (Table 2.3) and mass spectrometry. The $[M+1]^{+}$ peak at *m/z* 595 in the APCI-MS, indicated the molecular weight was equal to 594. Acid hydrolysis of compound 6 yielded a aglycon kaempferol. The sugars after acid hydrolysis were proved to be rhamnose and glucose by TLC with their standards. From the ^{13}C NMR, the C-3 of the aglycon showed a upfield shift by 2 ppm, indicated the glycosylation site was at C-3 position (Markham *et al.* 1978). Also the ^{13}C NMR showed the C-6 of the glucose shifted downfield by 6ppm, indicated a (1→6) linkage between the rhamnose and the glucose. The results of the ^{1}H NMR and ^{13}C NMR were compared with the data from Fico *et al.* (2000), proving that compound 6 was kaempferol-3-O-rutinoside (Figure 2.5).

2.5.7 Age-dependent changes in flavonoids and their glycosides

The present results showed that total flavonoids and their glycosides in the first month and the second month were almost similar. However, the total content of these flavonoids and their glycosides were increased by about 48% in the third month when compared with that in the second month.

Chapter 3

Hypolipidemic effects of soy leaves in hamsters

3.1 Introduction

3.1.1 Different lipoproteins and their functions

Most of the cholesterol in plasma is transported by 3 major lipoprotein classes: VLDL cholesterol (VLDL-C), LDL cholesterol (LDL-C), and HDL cholesterol (HDL-C) (Superko 2001). Triglyceride-rich VLDL, one of the major triglyceride carriers in the blood, is synthesized and secreted from the liver. After a series of interactions with the enzyme lipoprotein lipase, the particles become more dense and relatively cholesterol rich. An intermediate density lipoprotein (IDL) precedes the appearance of LDL, which is normally the greatest source of cholesterol transport among the lipoproteins. The role of LDL is for transporting cholesterol from liver to the peripheral tissues. In contrast to LDL, the HDL synthesized in liver is the main vehicle for the transfer of cholesterol from plasma and the peripheral tissues back to the liver for catabolism (Eckardstein *et al.* 1998, 2001; Stein and Stein 1999; McNamara 2000).

3.1.2 Risk factors of cardiovascular disease

Over the past 40 years, epidemiologic studies showed that blood levels of total and LDL-C are directly related to cardiovascular disease (LaRosa *et al.* 1990; Carleton *et al.* 1991). Cholesterol accumulation in the artery is accompanied by the development of atherosclerosis (Brown and Goldstein 1986). However, high level of total cholesterol in plasma may not be absolutely related to the cardiovascular disease (Hamilton 1997). LDL is the major cholesterol carrier in the blood, its oxidation is related to the formation of atherosclerosis (Parhami *et al.* 1993; Diaz *et al.* 1997; Young *et al.* 2001) (The role of LDL oxidation on the formation of atherosclerosis will be discussed in Chapter 4). As the amount of LDL cholesterol in the blood increase, the chance of being oxidized will be increased. Therefore, the amount of LDL cholesterol present in the blood can be used as one of the index for the risk of cardiovascular disease (Hamilton 1997; Ballantyne 1998).

Some other more precise indexes for the risk of cardiovascular disease are the ratios of LDL-C to HDL-C or non-HDL-C to HDL-C. Many studies showed that HDL-C was inversely related to the risk of cardiovascular disease (Backer *et al.* 1998; Boden *et al.* 2000a, 2000b; Despres *et al.* 2000). This may be due to the reversal cholesterol transport of HDL from peripheral cells and plasma back to the liver for catabolism (Eckardstein *et al.* 1998, 2001; Kwiterovich 1998; Philips *et al.* 1998).

As a result, the effect of reversal cholesterol transport of HDL can prevent the accumulation of cholesterol in plasma and decrease the chance of atherosclerosis.

3.1.3 Animal model

Golden syrian hamster (*Mesocricetus Auratus*) is a common model for hypocholesterolemic and cholesterol metabolism studies (Sugiyama *et al.* 1995; Campos *et al.* 1998; Terpstra *et al.* 1998; Trautwein *et al.* 1998, 1999; Ntanios and Jones 1999; Schneider *et al.* 2000). Similar to that in humans, the major cholesterol carrier in hamster is LDL (Nistor *et al.* 1987). It was found that about 50% of hamster's plasma cholesterol occurs in the LDL fraction (Nistor *et al.* 1987). Also hamsters are similar to humans in biliary sterol secretion (Spady and Dietschy 1984; Bocan and Guyton 1985). Rat is another common laboratory testing animal, however, they lack the cholesteryl ester transfer protein and are resistant to developing atherosclerosis due to high cholesterol feeding, while hamsters are like humans and are susceptible to atherosclerosis (Bok *et al.* 1999).

3.2 Objectives

The objective of the present study was to examine the hypolipidemic effect of soy leaves. Soybean has been shown to reduce plasma cholesterol in humans (Anthony *et al.* 1998; Nilausen and Meinertz 1998; Potter *et al.* 1998; Wong *et al.* 1998). However, the hypolipidemic effects of soy leaves have not been examined. In the present study, the hypolipidemic effects of soy leaves was investigated by using hamster as an animal model.

3.3 Materials and Methods

3.3.1 Animals

Male Golden syrian hamsters were housed (3- 4 hamsters per cage) in an animal room at 25 °C with 12:12-h light-dark cycles. Fresh semi-synthetic diets were given daily, and uneaten food was discarded. Food intake was measured daily and body weight was recorded twice a week. The hamsters were allowed access to food and fluid *ad libitum.*

Hamsters (125- 140 g) were randomly divided into 3 groups (n= 12). They were fed a semi-synthetic diet, with soy leaves powder (SLP) or soy leaves ethanol extract (SLEE) supplementation. The method described by Sanders & Sandaradura (1992) was modified and used to prepare the semi-synthetic diet. For the control group, diet was prepared by mixing the powdered ingredients (casein, 200 g; lard, 100 g; coconut oil, 100 g; starch, 418 g; sucrose, 100 g; mineral mix, 40 g; vitamin mix, 20 g; DL-methionine, 1 g; and cholesterol, 1 g) with 200 mL gelatin solution (100g/L). For the second and the third group of hamsters, 3% by weight soy leaves powder (SLP) and the ethanol extract (SLEE) derived from 3% soy leaves were mixed with the control diet respectively before setting them in gelatin. Once the gelatin had set, the diet was cut into approximately 20 g cubic portions and stored frozen (-20 °C). All the fecal samples were collected from each cage of the

hamsters at the end of each week. At the end of 4 weeks, all the hamsters were sacrificed after overnight fasting. Blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1500 g for 10 minutes and plasma was collected. The liver and perirenal adipose tissue were removed, washed with saline, and stored at –80 °C.

3.3.2 Serum lipid and lipoprotein determinations

Serum total triacylglycerols (TG) and total cholesterol (TC) levels were determined using enzymatic kits (Sigma Chemical, St. Louis, MO, USA). High-density lipoprotein cholesterol (HDL-C) was measured after precipitation of LDL and very low-density lipoprotein (VLDL) with phosphotungstic acid and magnesium chloride (Sigma).

3.3.3 Determination of cholesterol in the liver and adipose tissue

The liver (300 mg) or adipose tissue (300 mg) samples and 1 mg stigmastanol, as an internal standard, was homogenized in 15 mL chloroform-methanol (2:1, v/v) and 3 mL saline. The chloroform-methanol phase was removed and dried down under a gentle nitrogen steam. After 1 hour mild hydrolysis with 5 mL NaOH in 90% ethanol at 90 °C, 1 mL of water and 6 mL of cyclohexane were added for

cholesterol extraction. The cyclohexane phase was evaporated to dryness under nitrogen, and cholesterol was converted to its TMS-ether derivative by a commercial TMS-reagent (dry pyridine-hexamethyldisilazane-trichlorosilane, 9: 3: 1, v/v/v, Sil-A regent, Sigma). After 1 hour at 60 °C, the mixture was removed under nitrogen. The TMS-ether derivative was dissolved in 600 µl of hexane, and after centrifugation, the hexane phase was transferred to a vial for gas-liquid chromatograph (GLC) analysis. The TMS-ether derivative was analyzed in a fused silica capillary column (SAC™-5, 30 m x 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA, USA) in a Shimadzu GC-14B GLC equipped with a flame-ionization detector (Shimadzu). Column temperature was set at 285 °C and maintained for 20 minutes. Helium was used as carrier gas at a head pressure of 22 psi. A typical GLC chromatogram of liver cholesterol is shown in Figure 3.1.

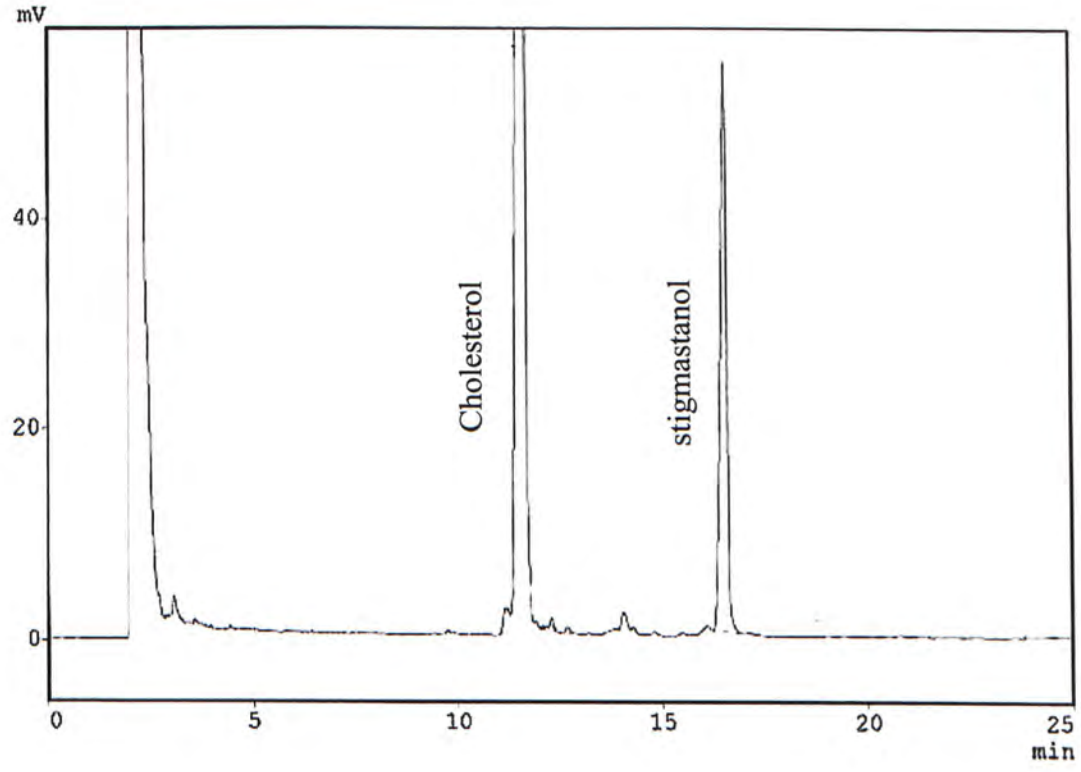


Figure 3.1 Gas liquid chromatographic profile of cholesterol in the liver

3.3.4 Extraction of neutral and acidic sterols from fecal samples

All the fecal samples were collected at the end of each week. The methods for the determination of fecal neutral and acidic sterols were according to Czubayko *et al.* (1991) with some modifications. Fecal samples were first dried in a lyophilizer and then grounded into powder form. Stigmasterol (0.5 mg in 1mL chloroform) was added into a tube as an internal standard for total neutral sterols. The tube was dried down under a gentle stream of nitrogen. Then 300 mg of grounded fecal sample and 0.5 mg hyodeoxycholic acid (0.5 mg in 2 mL 1 N NaOH) as an internal standard for total acidic sterols were added. The samples were then subjected to alkaline hydrolysis with 8 mL 1 N NaOH in 90% ethanol at 90 °C for 1 hour followed by cooling down to room temperature. Then 1 mL distilled water and 8 mL cyclohexane were added for extraction. After centrifugation, the upper cyclohexane phase and the lower aqueous phase were collected for determination of neutral and acidic sterols respectively.

3.3.4.1 Determination of neutral sterols

The cyclohexane phase was evaporated to dryness under a gently stream of nitrogen. The neutral sterols were converted to their TMS-ether derivatives by adding TMS-reagent (dry pyridine-hexamethyldisilazane-trichlorosilane, 9: 3: 1,

v/v/v, Sil-A regent, Sigma) and heated at 60 °C for 1 hour. The mixture was then dried down under nitrogen stream and the TMS-derivatives of neutral sterols were dissolved in 400 μL of hexane. After centrifugation, the hexane layer was transferred to a vial for GLC analysis.

3.3.4.2 Determination of acidic sterols

One mL of 10 N NaOH was added to the lower aqueous phase and the mixture was heated for 3 hours at 120 °C. Then 3 mL of distilled water was added to the mixture and cooled to room temperature. The mixture was acidified with 1 mL 25% HCl. And the acidic sterols were extracted with 7 mL diethyl ether for two times. The ether phases were pooled together and then dried down under nitrogen stream. Methylation of the acidic sterols was performed by adding 2 mL methanol, 2 mL dimethoxypropane and 40 μL concentrated HCl. The mixture was mixed thoroughly and allowed to stand at room temperature for overnight. The acidic sterols were then dried down under nitrogen stream. TMS-reagent was added and then heated at 60°C for one hour to convert the acidic sterols into their TMS-ether derivatives. The mixture was dried down under nitrogen stream and the TMS-derivatives of neutral sterols were dissolved in 300 μL of hexane. After centrifugation, the hexane layer was transferred to a vial for GLC analysis.

3.3.4.3 GLC analysis of neutral and acidic sterols

The analysis of fecal neutral and acidic sterols was carried out in a GLC equipped with a fused silica capillary column as described in part 3.3.3. For the neutral sterols, the column temperature was set at 285 °C and maintained for 30 min. For the acidic sterols, the column temperature was programmed from 230 to 280 °C at a rate of 1 °C/ min. Helium was used as a carrier gas at a head pressure of 22 psi in both neutral and acidic sterol analysis. Typical chromatograms of neutral and acidic sterols were shown in Figures 3.2 and 3.3, respectively.

3.3.5 Statistics

Student's t-test and one-way analysis of variance (ANOVA) were used for statistical evaluation of differences between groups (SigmaStat version 2.01, SigmaStat Advisory Statistical Software, MO, USA)

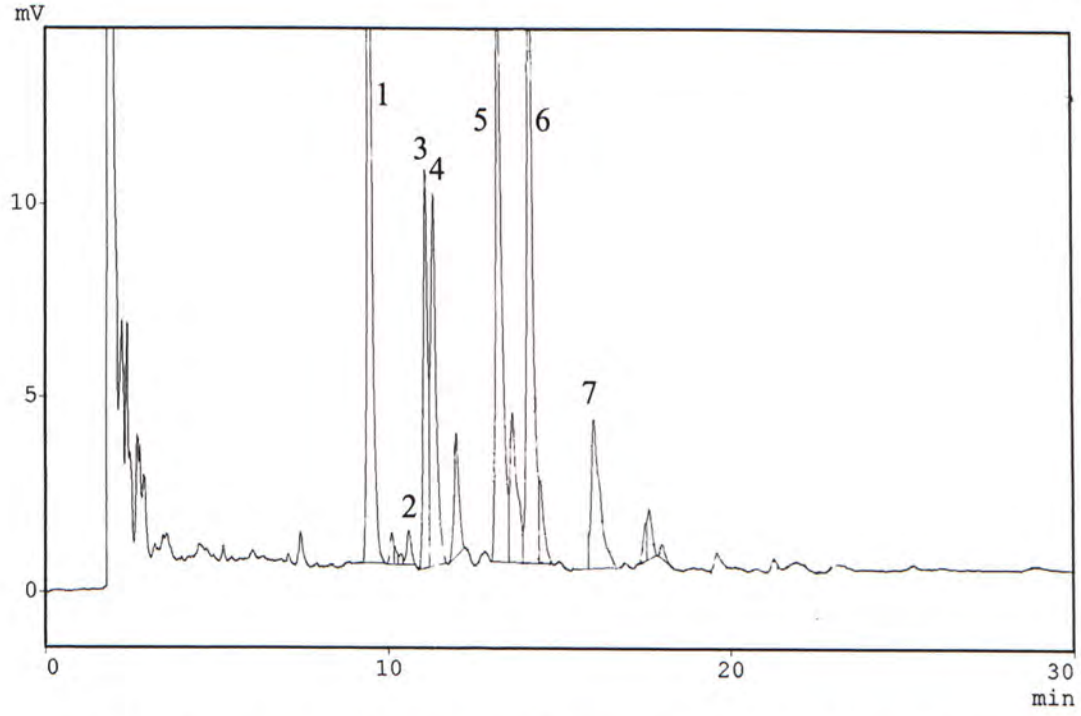


Figure 3.2 Gas liquid chromatographic profile of neutral sterols in feces. Identification of peaks: 1, coprostanol; 2, coprostanone; 3, cholesterol; 4, dihydrocholesterol; 5, campersterol; 6, stigmasterol (internal standard) and 7, β-sitosterol

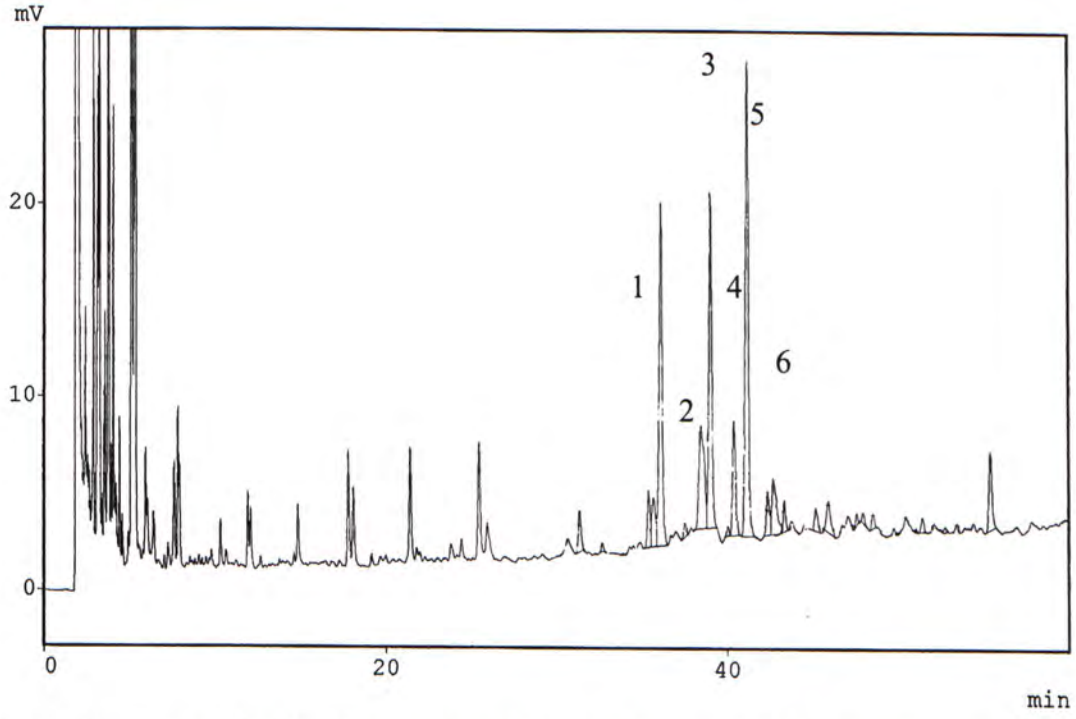


Figure 3.3 Gas liquid chromatographic profile of acidic sterols in feces. Identification of peaks: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxcholic acid; 4, cholic acid; 5, hyodeoxycholic acid (internal standard) and 6, ursodecholic acid.

3.4 Results

3.4.1 Growth and food intake

The body weight gain and food intake of hamsters are shown in Table 3.1. No significant differences in body weight and food intake were observed among the control, SLP and SLEE (Table 3.1).

3.4.2 Effects of SLP and SLEE supplementation on serum triacylglycerol (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C)

After hamsters were fed a high-fat and high-cholesterol diet for 4 weeks, their serum triacylglycerol, total cholesterol and HDL-C were measured. There were no significant differences in serum TG and TC among the three groups (Table 3.2).

A significant increase in HDL-C by 10.9% was observed in the SLEE group ($p < 0.05$) compared with the control group. However, no difference was observed between the SLP and control group (Table 3.2).

3.4.3 Effects of SLP and SLEE supplementation on non-HDL-C and ratio of non-HDL-C to HDL-C

Non-HDL-C was defined as a difference between TC and HDL-C. Non-HDL-C levles for the control, SLP and SLEE groups were 87.8 ± 5.4, 77.3 ± 13.9 and 80.1 ± 12.3 mg/dL respectively. A significant reduction of non-HDL-C by 12% and 8.8% compared with that in the control was observed in the SLP ($p < 0.05$) and SLEE ($p < 0.05$) groups respectively (Table 3.2).

When data were expressed as the ratio of non-HDL-C to HDL-C, it was found that the SLP and SLEE groups were significantly different from the control (Table 3.2).

Table 3.1 Effects of SLP and SLEE supplementations on body weight and food intake in hamsters

	Control	SLP	SLEE
Initial body wt (g)	132.5 ± 5.1	132.7 ± 3.9	132.4 ± 5.1
Final body wt (g)	146.3 ± 7.6	148.8 ± 5.0	149.1 ± 8.5
Liver wt (g)	4.9 ± 0.4	5.0 ± 0.4	5.1 ± 0.5
Food intake (g)	6.6 ± 0.4	6.4 ± 0.8	6.5 ± 0.2

Data are expressed as mean ± S.D. of n= 12

SLP: Soy leaves powder group

SLEE: Soy leaves ethanol extract group

Table 3.2 Effects of SLP and SLEE supplementations on serum TG, TC, HDL-C, non-HDL-C and the ratio of non-HDL-C to HDL-C

	Control	SLP	SLEE
TG (mg/dL)	203.3 ± 52.2	200.8 ± 47.2	228.8 ± 40.3
TC (mg/dL)	183.6 ± 9.3	174.9 ± 23.2	184.8 ± 11.0
HDL-C (mg/dL)	95.8 ± 7.0[a]	97.52 ± 11.4	104.8 ± 10.4[b]
Non-HDL-C (mg/dL)	87.83 ± 5.4[a]	77.3 ± 13.9[b]	80.1 ± 12.3[b]
Non-HDL-C/ HDL-C	0.92 ± 0.08[a]	0.79 ± 0.11[C]	0.78 ± 0.18[C]

Data are expressed as mean ± S.D. of n= 12

SLP: Soy leaves powder group

SLEE: Soy leaves ethanol extract group

Non-HDL-C = TC – HDL-C

Means at the same row with different superscripts (a, b) differ significantly ($p< 0.05$)

Means at the same row with different superscripts (a, c) differ significantly ($p< 0.01$)

3.4.4 Effects of SLP and SLEE supplementations on concentration of hepatic cholesterol

The liver weights of the hamsters were measured immediately after the scarification, no significant differences were found among all the three groups (Table 3.1). Also, there was no observable difference for the morphology and the color of the liver between the control and the treatment groups. The hepatic cholesterol contents in the control, SLP and SLEE groups were 24.9 ± 4.1, 30.4 ± 4.2 and 30.3 ± 2.8 mg/ g of liver respectively. It was found that the hepatic cholesterol contents in SLP ($p< 0.01$) and SLEE ($p< 0.01$) groups were significantly higher than the control group (Figure 3.4).

3.4.5 Effects of SLP and SLEE supplementations on perirenal adipose tissue cholesterol

There was no significant difference in perirenal adipose tissue cholesterol content among the three groups (Figure 3.5).

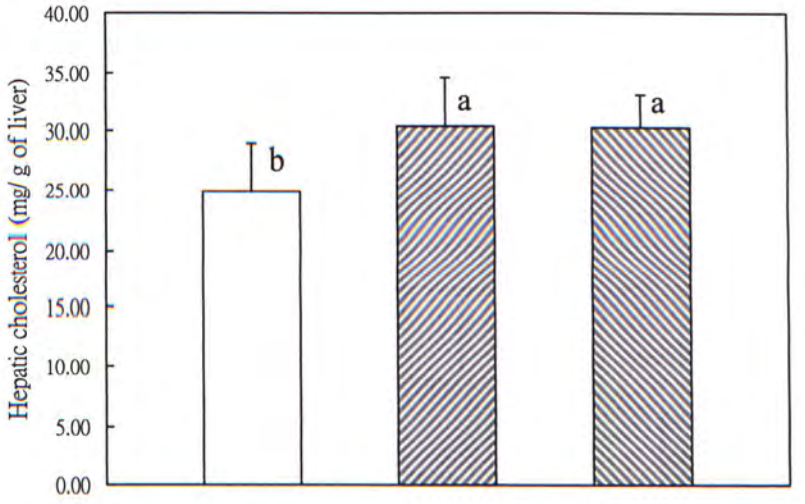


Figure 3.4 Effects of soy leaves powder (SLP) and soy leaves ethanol extract (SLEE) on concentration of hepatic cholesterol in hamsters. Data are expressed as means ± S.D. of n= 12. Means with different superscript letter (a, b) differ significantly at $p< 0.01$

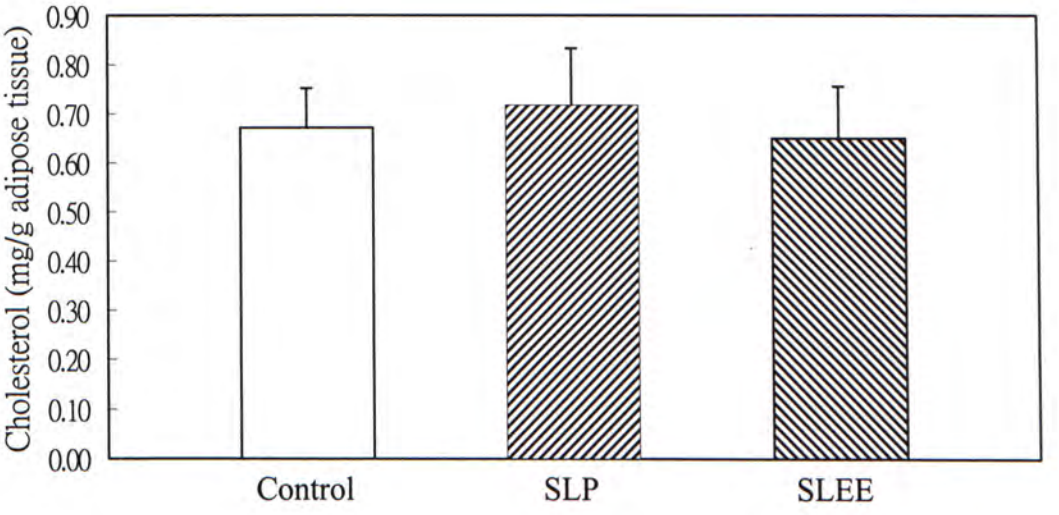


Figure 3.5 Effects of soy leaves powder (SLP) and soy leaves ethanol extract (SLEE) on perirenal adipose tissue cholesterol. Data are expressed as mean ± S.D. of n= 12

3.4.6 Effects of SLP and SLEE supplementations on fecal neutral and acidic sterols

The neutral sterols refer to the sum of coprostanol, coprostanone, cholesterol, dihydrocholesterol, campersterol, and β-sitosterol. The total fecal neutral sterols were significantly elevated in the SLP group compared with that in the control. Supplementation of SLEE in the diet slightly increased the excretion of total neutral sterols but no statistical differences were observed between the SLEE hamster and the control group (Figure 3.6).

The acidic sterols measured include lithocholic, deoxycholic, cholic and ursodeoxycholic acid. Supplementation of SLP and SLEE did not affect the fecal excretion of acidic sterols as compared with the control except at week 2 when statistical difference was found between the SLP group and the control hamsters (Figure 3.7).

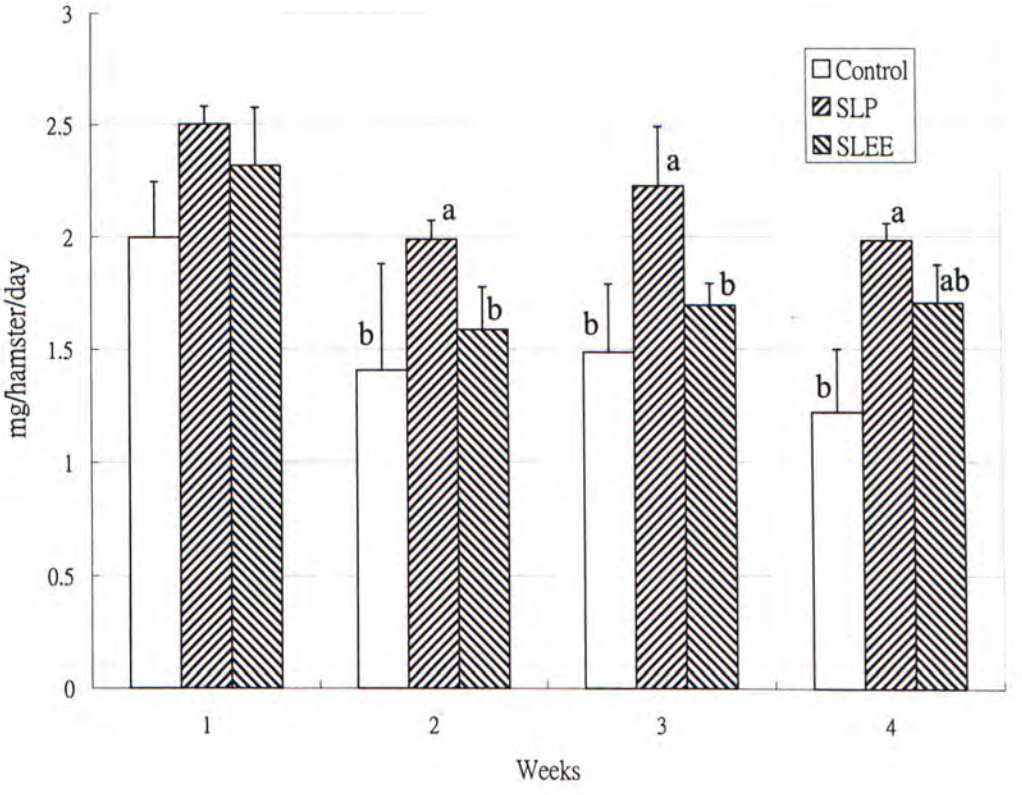


Figure 3.6 Effects of SLP and SLEE supplementations on concentration of fecal neutral sterols. Data are expressed as mean ± S.D. of n= 4 cages. Means with different superscript letter (a, b) differ significantly at $p< 0.05$. SLP: Soy leaves powder group; SLEE: Soy leaves ethanol extract group.

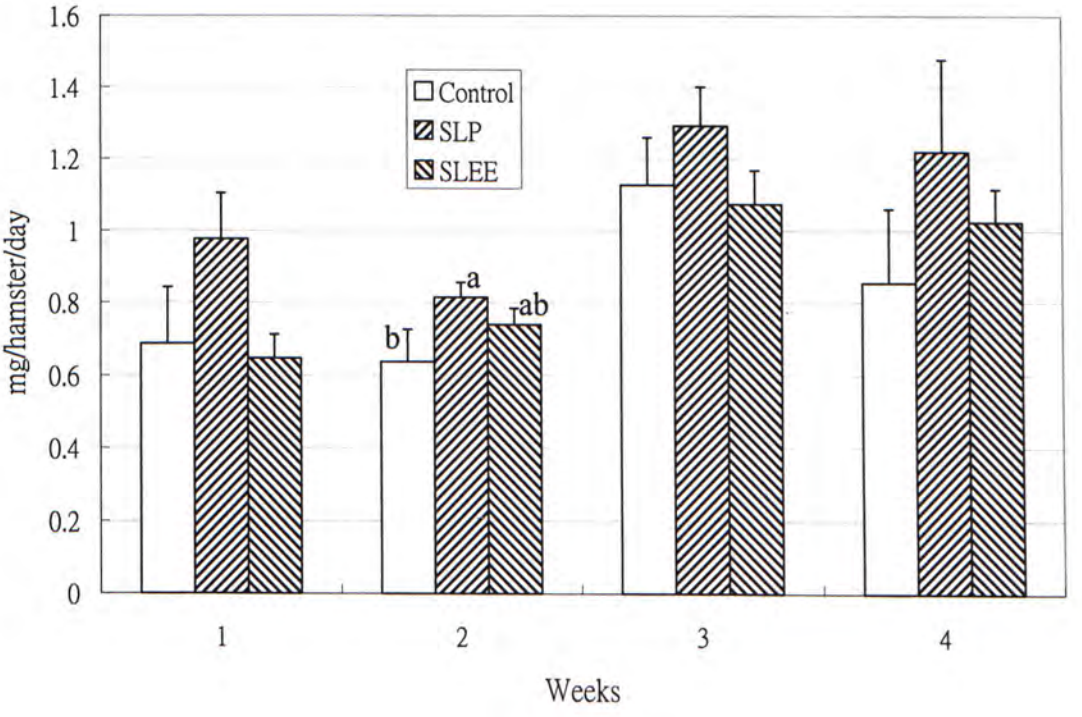


Figure 3.7 Effects of SLP and SLEE supplementations on the concentration of fecal acidic sterols. Data are expressed as mean ± S.D. of n= 4 cages. Means with different superscript letter (a, b) differ significantly at $p< 0.05$. SLP: Soy leaves powder group; SLEE: Soy leaves ethanol extract group.

3.5 Discussion

The present study demonstrated that supplementation of soy leaves in the diet could not decrease serum total TG and TC. However, there was a significant decrease in the ratio of non-HDL-C to HDL-C in the SLP and SLEE groups, suggesting that supplementation of SLP and SLEE could cause the redistribution of serum cholesterol. This ratio is commonly used as a risk factor in cardiovascular disease. The higher the ratio, the greater the chance of getting cardiovascular diseases. If the data could apply to humans, the decrease in the ratio of non-HDL-C to HDL-C indicated that soy leaves supplementation in the diet might be associated with a decreased risk of cardiovascular diseases.

The effect of SLP and SLEE on serum lipoprotein profile may not be fully due to the effect of dietary fiber. First, both SLP and SLEE groups had a significantly lower ratio of non-HDL-C to HDL-C compared with the control. However, only SLP diet contained fiber while SLEE did not had any fiber. Second, the effect of dietary fiber on serum cholesterol is different from the observation in the present study. From a meta-analysis of the cholesterol-lowering effects of dietary fiber, Brown *et al.* (1999) reviewed that dietary fiber can generally decrease the total serum cholesterol with the HDL-C being unaffected.

Previous studies showed that supplementation of soy-protein could significantly

increased the HDL-C and decreased the ratio of LDL-C to HDL-C, but total serum cholesterol was not significantly reduced in humans (Nilausen and Meinertz 1998). This is in agreement with the result in the present study, implying that some of the common components present in soy leaves and soybean might be responsible for hypolipidemic activity of soy products. A previous study showed that supplementation of soy isoflavones in the diet could decrease non-HDL-C and increase HDL-C (Anthony *et al.* 1998). Since, genistein can bind to estrogen receptors (Kuiper *et al.* 1998) to exert estrogenic activity and may share the LDL reducing and HDL increasing effect of human estrogen (Lilley *et al.* 1998; Westerveld 1998; Godsland 2001). Therefore, soy isoflavones may be one of the active ingredients which were responsible for the observed effect.

The mechanisms by which SLP and SLEE lowered the ratio of non-HDL-C to HDL-C remained unexplored. One of the possible mechanisms may be associated with increased fecal excretion of neutral and acidic sterols. Another possibility is that supplementation of SLP and SLEE increased the catabolism of cholesterol in the liver. The present results showed that there was an increase in the hepatic cholesterol in the SLP and SLEE groups. This might be due to the increase in serum HDL-C that increased the rate of cholesterol transported from the peripheral parts of the body back to the liver for degradation. However, the rate of cholesterol

metabolized by the liver to form the acidic sterols might not as high as the rate of cholesterol entering into the liver, leading to elevation in hepatic cholesterol (Figure 3.4).

Chapter 4

Effects of soy leaves and its flavonoid glycosides on haemolysis and on LDL oxidation

4.1 Introduction

In recent years, there has been a great interest in health effects of dietary antioxidants against coronary heart disease (Parthasarathy *et al.* 1998). The underlying cause of coronary heart disease, strokes and peripheral arterial disease is mainly atherosclerosis. It is responsible for about half the deaths in the developed countries and its incidence in developing countries is increasing.

The major harmful effect of atherosclerosis is on the large and medium-sized arteries. The arterial wall has three layers namely innermost layer, tunica intima, tunica media and tunica adventitia. The intima is normally a thin layer but becomes greatly thickened when an atherosclerotic lesion forms. The formation of atherosclerotic lesions may last over a period of years or decades, but their bulk can impede the flow of blood through the arteries. Most heart attacks (myocardial infarctions) are due to the sudden fissuring of an atherosclerotic lesion. These fissures cause blood to come into contact with substances inside the lesions that cause a thrombus and block the flow of blood (Wilcox *et al.* 1995), leading to a

myocardial infarction if the artery supplies the muscles of the heart.

4.1.1 Role of low density lipoprotein oxidation in the development of atherosclerosis

The oxidative theory of atherosclerosis proposes that it is not LDL itself but oxidized LDL that is atherogenic in the arterial wall (Diaz *et al.* 1997 and Young *et al.* 2001). LDL enters the arterial wall from the plasma and accumulates in the extracellular subendothelial space of arteries and, through the action of resident vascular cells, is mildly oxidized to a form known as minimally modified LDL. This minimally modified LDL induces local vascular cells to produce monocyte chemotatic protein, which stimulates monocyte recruitment and differentiation to macrophages in arterial walls (Parhami *et al.* 1993). The accumulating monocytes and macrophages stimulate further peroxidation of LDL.

In contrast to the uptake of native LDL by the LDL receptor on macrophages, the uptake of oxidized LDL by the scavenger-receptor pathway is not subject to negative-feedback regulation. The aldehydes formed during LDL oxidation can combine with the epsilon-amino groups of the lysyl residues of apo B-100 to form Schiff bases. When a Schiff base forms, the normal positive charge of the lysyl residue is abolished and the LDL particles acquire a greater negative charge

(Steinbrecher 1987). As a result, the oxidized LDL can no longer be recognized by the native LDL receptor and thus results in massive uptake of cholesterol (from oxidized LDL) by the macrophages (Diaz *et al.* 1997). The amount of cholesterol in LDL entering the cells thought to overwhelm the capacity of the macrophages to release it and the cholesterol therefore accumulate inside the cells, converting them into foam cells Figure 4.1. Oxidized LDL also has direct chemotactic activity for monocytes and stimulates the binding of monocytes to the endothelium. The monocytes will become trapped in the subendothelial space, if they cross the endothelial layer. This is because that oxidized LDL can inhibit their egress from the arterial wall (Quinn *et al.* 1987).

In the later lesions, deposition of extracellular cholesterol esters forms a large pool of lipids at the base of the lesions. Then proliferation of smooth muscle cells in the intima will occur and these smooth muscle cells will secrete large amounts of collagen, which adds bulk to the lesions. The macrophages die in the deeper parts of the lesions, mainly at the edge of the lipid pool in advanced lesions. Therefore, oxidation of LDL is highly related to atherosclerosis.

4.1.2 LDL oxidation

LDL is subject to oxidation in many cell types, including monocytes, macrophages, neutrophils, endothelial cells, smooth muscle cells and fibroblasts. However, the cell types that are mainly responsible for the oxidation of LDL and atherosclerosis are macrophages, endothelial cells and smooth muscle cells (Yong *et al.* 2001). The mechanisms by which these cells oxidize LDL are poorly understood. Involvement of superoxide (Steinbrecher *et al.* 1988 and Hiramatsu *et al.* 1987) and lipoxygenase (Parthasarathy *et al.* 1989 and Rankin *et al.* 1991) have been proposed. Another proposed mechanism was that cells might oxidize LDL by releasing the thiol-containing amino acid cysteine (Heinecke 1987). Oxidation of cysteine led to form cystine in the extracellular space, producing sulf- or oxygen centered free radicals that may attack LDL. When cysteine is oxidized to cystine, iron (III) may be reduced to iron (II) while copper (II) is converted to copper (I). These transition metal ions in their valency states may oxidize LDL better than in their higher valency states (Heinicke *et al.* 1987).

Whatever the mechanism is, oxidation of LDL will involve the abstraction by an unidentified free radical of a hydrogen atom from a methylene (CH_2) group of a polyunsaturated fatty acid (PUFA). Molecular rearrangement of the resulting unstable carbon radical results in a more stable configuration, a conjugated diene.

The conjugated diene reacts very quickly with molecular oxygen, and the peroxyl radical thus formed a crucial intermediate. The PUFA peroxyl radical in LDL may abstract a hydrogen atom from an adjacent PUFA to form a hydroperoxide and another lipid radical, a reaction which results in chain propagation. The fragments of lipid hydroperoxides will eventually break down into shorter-chain aldehydes and other products, including malondialdehyde (MDA) and 4-hydroxynonenal (Figure 4.2) (Young *et al.* 2001)

4.1.3 Thiobarbituric acid reactive substances (TBARS) as an index of LDL oxidation

Measurement of TBARS formation is a most frequently used test as an index of lipid peroxidation. The breakdown product of lipid peroxidation, malondialdehyde (MDA), is formed when lipid hydroperoxide is decomposed during LDL oxidation (Figure 4.2). These MDA molecules react with thiobarituric acid (TBA) in acidic condition to form a pink chromagen, which has an absorption maximum at 532 nm. The amount of these chromagen can be quantified by spectrophotometrically or fluorometrically (Ohkawa *et al.* 1978). This method is sensitive to determine the degree of lipid peroxidation.

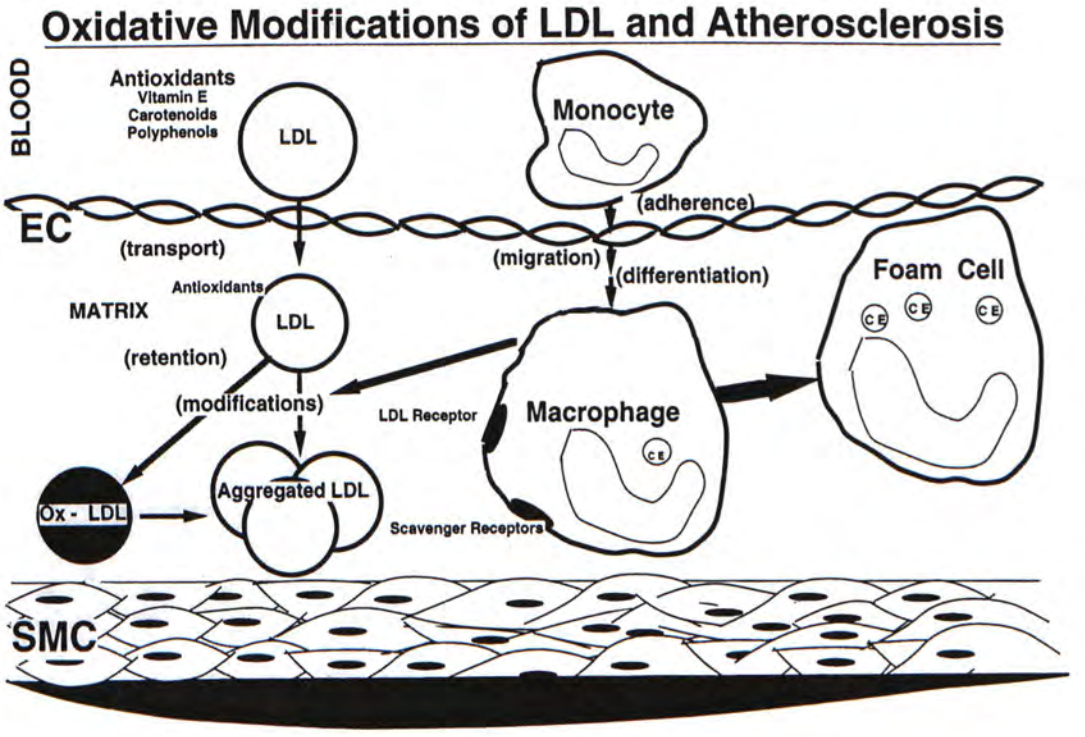


Figure 4.1 Macrophage-mediated oxidation and aggregation of LDL, formation of foam cells (Adapted from Aviram *et al.* 1998)

Native LDL particles migrated into the extracellular subendothelial space and then oxidized by the adjacent cells. The oxidized LDL was taken up by the macrophages. Cholesterol esters in oxidized LDL enter the macrophages and overwhelm their capacity to release them. These cholesterol esters therefore accumulate inside the cells, converting them into foam cells.

CE: Cholesterol ester
SMC: Smooth muscle cell
Ox-LDL: Oxidized LDL

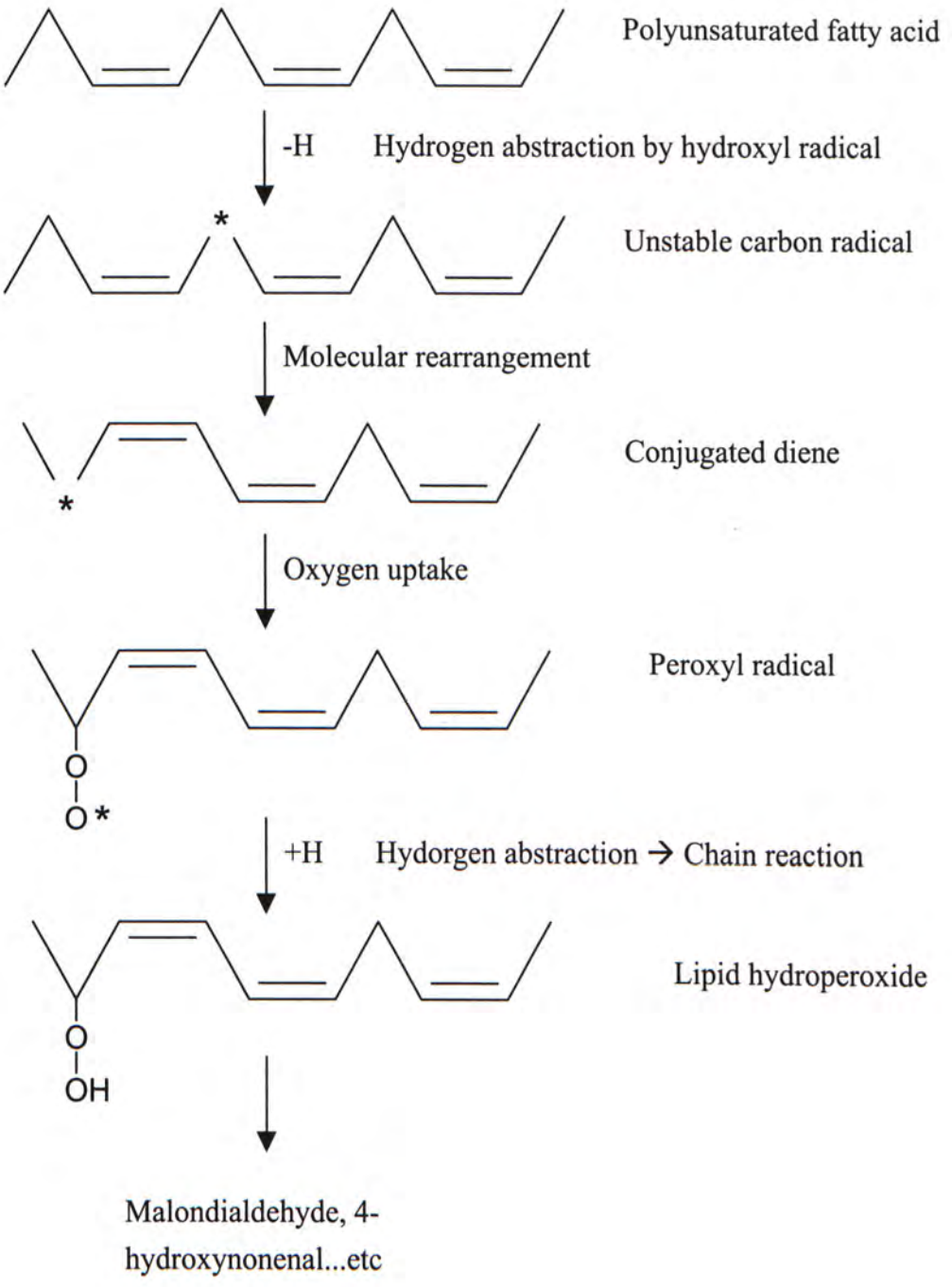


Figure 4.2 Basic reaction sequence of lipid peroxidation (Adapted from Young 2001 with some modifications)

4.1.4 Antioxidant and LDL oxidation

The major lipid-soluble antioxidant present in LDL is vitamin E (α-tocopherol). Enrichment of vitamin E has been shown to retard LDL oxidation and inhibit the proliferation of smooth muscle cells (Chan 1998).

Some natural antioxidants, for example, flavonoids may protect vitamin E in LDL, either by scavenging free radicals themselves or by regenerating vitamin E from its radical form (Zhu *et al.* 1999). Some studies showed that a mixture of flavonoid, α-tocopherol and ascorbic acid might inhibit LDL oxidation synergistically (Negre *et al.* 1995 and Hwang *et al.* 2000).

4.2 Objective

The objective of the present study was to examine the antioxidative effects of three different soy leaves extracts and their flavonoid glycosides by using Cu^{2+}-mediated human LDL oxidation and inhibition on haemolysis as two different assay models. The possible mechanism of their antioxidative effects was also determined.

4.3 Materials and methods

4.3.1 Isolation of LDL from human serum

Fresh human serum was collected from healthy subjects at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To prevent lipoprotein modification, EDTA and NaN_3 were added to the serum collected (final concentrations of EDTA and NaN_3 were 0.1% and 0.05%, respectively). LDL was isolated from the serum according to the method described previously (Havel *et al.* 1995). In order to prevent LDL from oxidation, the centrifuge tubes containing serum were flushed with nitrogen gas. Firstly, the serum was centrifuged at 1500 g for 15 minutes to remove cells and cell debris. NaCl-KBr solution (dissolve 153 g NaCl, 354 g KBr and 100 μg EDTA in one liter of water, 1.33 g/mL) was then added to increase the density to 1.019. The serum was re-centrifuged at 160,000 g for 20 hours at 4 °C. After the removal of the top layer containing chylomicron and very low-density lipoprotein (VLDL), the density of remaining serum fractions was increased to 1.064 and re-centrifuged at 160,000 g for an additional 24 hours at 4 °C. The top LDL fraction was collected and then flushed with nitrogen and stored at –70 °C. The protein content of isolated LDL was determined using Lowry's method (Lowry *et al.* 1951).

4.3.2 LDL oxidation

The stock LDL fraction (5 mg protein/mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH = 7.4) containing 0.01 M sodium phosphate, 0.9 % NaCl, 10 μM EDTA and 0.05% NaN_3 in dark at 4 °C for 24 hours. The dialysis solution was changed four times. Oxidation of LDL was conducted as previously described by Puhl *et al.* (1994). LDL protein (100 μg) was incubated in a mixture containing 5 μM $CuSO_4$ at 37 °C for up to 24 hours. The oxidation of LDL was then stopped by addition of 25 μL 1.0% EDTA and cooled at 4 °C.

4.3.3 Determine the formation of thiobarbituric acid-reactive substances (TBARS)

The degree of LDL oxidation was monitored by measuring the production of TBARS as previously described (Buege & Aust 1978). After the reaction was stopped by addition of EDTA at 4 °C, 2 mL of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCl solution were added to the LDL-incubated tube. The incubation mixture was then heated at 95 °C for 1 hour, cooled on ice, and centrifuged at 1000 g for 20 minutes. The formation of TBARS was determined by measuring the absorbance at 532 nm. Calibration was done with a malondialdehyde (MDA) standard solution prepared from tetramethoxylpropane. The extent of LDL

oxidation was expressed as nmol MDA/mg LDL protein.

4.3.4 Assay for erythrocyte haemolysis

The erythrocyte hemolysis assay was performed according to the procedures described by Ng *et al.* (2000) with some modifications.

Fresh rabbit blood was collected from the New Zealand white rabbits and kept at 4 °C before use. Erythrocytes were separated by centrifugation at 1500 g for 20 minutes at 4 °C. The erythrocytes were then washed three times with 10 volumes of 10mM phosphate buffered saline (PBS) which was prepared by mixing, 2.88 g $Na_2HPO_4.2H_2O$, 0.488 g, NaH_2PO_4 and 18 g NaCl in 2000 mL H_2O at pH 7.4. During the last washing, the erythrocytes were centrifuged at 1500 g for 10 minutes to obtain a constantly packed cell preparation. The packed erythrocytes were diluted to 20% of the original concentration by using PBS. One milliliter of 200 mM 2, 2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) (Wako, Japan) in PBS, 0.5 mL test substance or 0.5 mL PBS as control and 1 mL erythrocytes suspension were mixed and incubated at 37 °C for 2.5 hr. After incubation, 4 mL PBS was added into the reaction mixture. The diluted mixture was centrifuged at 1000 g for 10 min. Absorbance (A) of the supernatant was measured at 540 nm. Percentage inhibition of haemolysis was calculated by the equation: % inhibition=

$(1 - A_{test}/ A_{control}) \times 100\%$. Epigallocatechin gallate (EGCG) was used as a positive control.

4.3.5 Statistics

Results were expressed as mean ± standard deviation (S.D.). Student's t-test and one-way analysis of variance (ANOVA) were used for statistical evaluation of differences between groups (SigmaStat version 2.01, SigmaStat Advisory Statistical Software, MO, USA)

4.4 Results

4.4.1 Effects of three different soy leaves extracts and flavonoid glycosides on LDL oxidation

LDL was oxidized significantly within 2 hours in the control. Six different kaempferol glycosides at a concentration of 40 μM showed no or very little protection to LDL from Cu^{2+} -mediated oxidation (Figure 4.3). LDL was oxidized completely within 4, 6 and 8 hours in the presence of 0.1 mg/mL soy leaves water, chloroform and butanol extracts respectively (Figure 4.4). LDL was also oxidized completely within 4 hours in the presence of 40 μM genistein (Figure 4.4). On the other hand, the positive control LDL in the presence of 40 μM EGCG, a major antioxidant present in green tea, did not show any oxidation throughout the period of 24 hours incubation (Figure 4.4).

4.4.2 Effects of three soy leaves extracts and flavonoid glycosides on erythrocyte haemolysis

The water and chloroform extracts showed a very little inhibition on erythrocyte haemolysis at the concentration of 0.1 mg/mL (< 5%). The butanol extract, showed 10.7% and 37.0% inhibition on erythrocyte haemolysis at the concentrations of 0.05 and 0.1mg/mL respectively. The pure kaempferol glycosides at the

concentration of 20 μM and 40 μM exhibited inhibiton on erythrocyte haemolysis by > 20% and > 60% respectively. In contrast, inhibition of EGCG at 20μM and 40μM were found to be 38% and 83% respectively (Table 4.1).

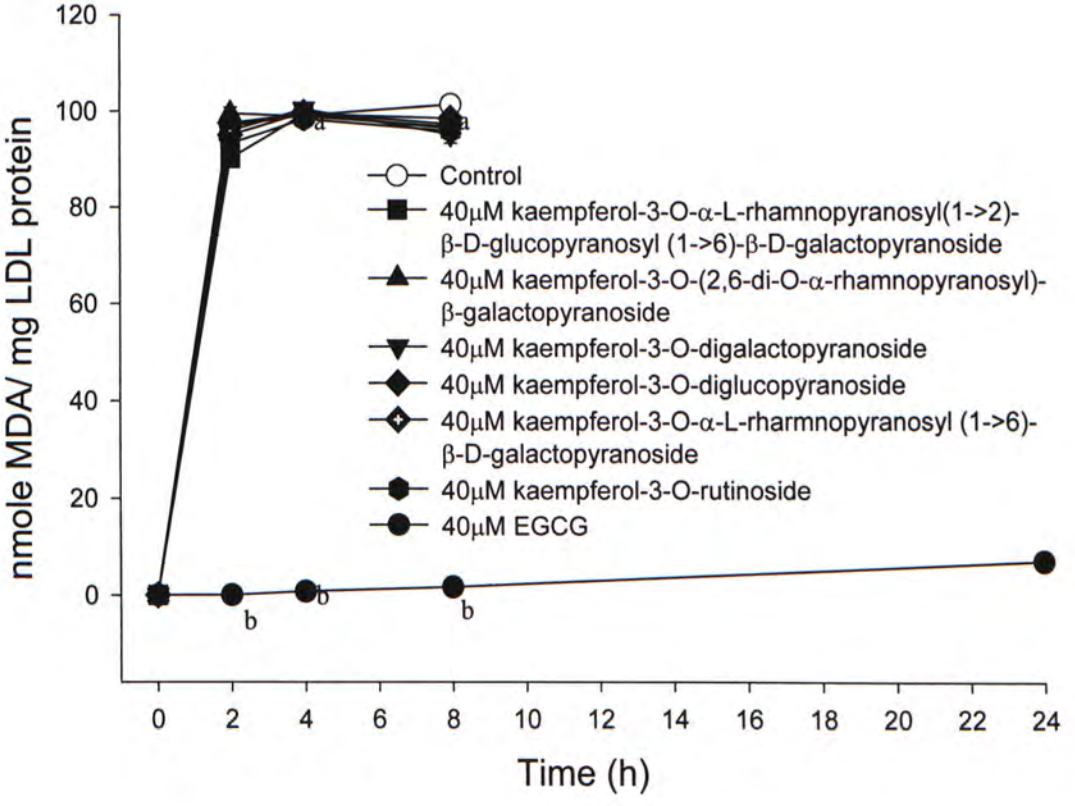


Figure 4.3 Inhibitory effects of six different kaempferol glycosides from soy leaves on the production of thiobarbituric acid reactive substances (TBARS) in Cu^{2+}-mediated oxidation of human LDL. Data are expressed as mean ± S.D. of n= 3. Means at the same time point with different letters (a- b) differ significantly from the control values ($p< 0.01$).

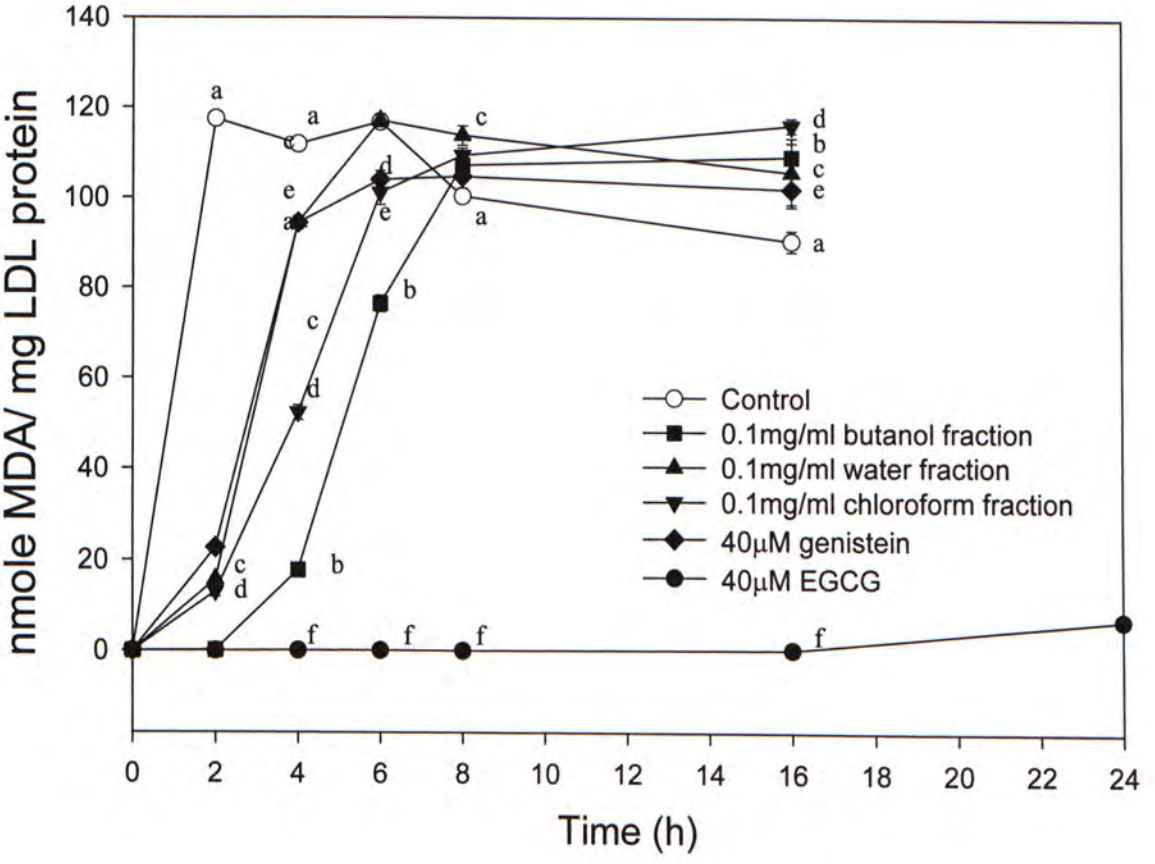


Figure 4.4 Inhibitory effects of three different soy leaves extracts on the production of thiobarbituric acid reactive substances (TBARS) in Cu^{2+}-mediated oxidation of human LDL. Data are expressed as mean ± S.D. of n= 3. Means at the same time point with different letters (a- f) differ significantly from the control values ($p< 0.01$)

EGCG : Epigallocatechin gallate

Table 4.1 Inhibitory effects of three different soy leaves extract and six different kaempferol glycosides from soy leaves on erythrocyte haemolysis.

	% inhibition
Control (PBS)	0.0 ± 0.2^{a}
20μM kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside	23.5 ± 0.4^{c}
20μM kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside	22.2 ± 0.4^{c}
20μM kaempferol-3-O-digalactopyranoside	22.6 ± 0.5^{c}
20μM kaempferol-3-O-diglucopyranoside	24.3 ± 0.6^{c}
20μM kaempferol-3-O-α-L-rharmnopyranosyl (1→6)- β-D-galactopyranoside	20.4 ± 0.5^{c}
20μM kaempferol-3-O-rutinoside	23.8 ± 0.4^{c}
EGCG (20μM)	38.5 ± 0.9^{c}
40μM kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside	60.8 ± 0.3^{c}
40μM kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside	68.4 ± 1.9^{c}
40μM kaempferol-3-O-digalactopyranoside	60.0 ± 0.7^{c}
40μM kaempferol-3-O-diglucopyranoside	69.8 ± 0.6^{c}
40μM kaempferol-3-O-α-L-rharmnopyranosyl (1→6)- β-D-galactopyranoside	62.2 ± 0.6^{c}
40μM kaempferol-3-O-rutinoside	69.2 ± 1.4^{c}
EGCG (40μM)	82.7 ± 1.6^{c}
Butanol extract (0.05mg/mL)	10.7 ± 0.7^{c}
Water extract (0.05mg/mL)	1.1 ± 0.8
Chloroform extract (0.05mg/mL)	1.7 ± 0.9^{b}
Butanol extract (0.1mg/mL)	37.0 ± 1.1^{c}
Water extract (0.1mg/mL)	3.7 ± 1.7^{b}
Chloroform extract (0.1mg/mL)	4.8 ± 0.5^{c}

Data are expressed as mean ± S.D. of n= 3.

Means at the same row with different superscripts (a, b) differ significantly ($p< 0.05$)

Means at the same row with different superscripts (a, c) differ significantly ($p< 0.01$)

EGCG: Epigallocatechin gallate

4.5 Discussion

Oxidative stress in the body can damage to macro-molecules like DNA, proteins and lipids, and might be involved in atherosclerosis (Holvoet *et al.* 1998 and Heinecke 1998), cancer (Keum *et al.* 2000; Mukhtar *et al.* 2000 and Steele *et al.* 2000) and chronic inflammation (Halliwell 1994). In atherosclerosis, for example, oxidation of low density lipoproteins is thought to play an important role (Hamilton 1997 and Young *et al.* 2001). It has been hypothesised that the flavonoid antioxidants may protect tissue against damage caused by free radicals (Aviram *et al.* 1998).

The present study demonstrated that kaempferol glycosides from soy leaves did not show any antioxidative effect on Cu^{2+}- mediated LDL oxidation. But soy leaves butanol, water and chloroform extracts showed a weak antioxidative effect on LDL oxidation. This indicated that the antioxidant present in these soy leaves extracts were not due to kaempferol glycosides. At the concentration of 0.1 mg/mL soy leaves butanol extract, the total concentration of genitein and its glycosides was 4.0 μM (Table 4.2). It was assumed that genistein and its glycosides at this concentration could not protect LDL from Cu^{2+}- mediated oxidation. Since genistein at the concentration of 40 μM, LDL was oxidized significantly within 4 hours. This proved that the antioxidative effect of soy leaves butanol extract was

not due to genistein and its glycosides. The present results indicated that the major antioxidant present in soy leaves butanol extract which was responsible for protecting LDL from Cu^{2+}- mediated oxidation has not been isolated.

Vitamin E (α- tocopherol) is a major lipid soluble antioxidant present in LDL (Anitra *et al.* 2000). Depletion of endogenous vitamin E would lead to LDL oxidation (Diaz *et al.* 1997). Regeneration of vitamin E by the secondary antioxidant can inhibit LDL oxidation and decrease the rate of atherosclerosis (Chan 2000 and Hamilton 1997). Jovanovic *et al.* (1998) showed the reduction poteintial of vitamin E was 0.48 V. Some flavonoids, epigallocatechin (0.42 V), epigallocatechin gallate (0.43 V) and querectin (0.33 V) (Jovanovic *et al.* 1998), their reduction potentials were smaller than viatmin E (0.48 V), therefore, they may repair vitamin E radical and maintaining desirable concentration of this important physiological antioxidant (Zhu *et al.* 1999 and Zhu *et al.* 2000). However, the reduction potential of kaempferol was found to be 0.75 V (Jovanovic *et al.* 1997) which was greater than vitamin E (0.48 V). Therefore, kaemfperol could not regenerate vitamin E and is unable to protect LDL from oxidation through this mechanism.

Haemolysis assay showed that kaempferol glycosides from soy leaves could significantly inhibit erythrocyte haemolysis in a dose-dependent manner. This may be due to the effect of flavonoids on the inactivation of superoxide radical and singlet oxygen generated by AAPH. Jovanovic *et al.* (1997 and 1998) showed the reactivities of flavonoids and gallocatechins with the superoxide radical were expected from their excellent electron donating abilities, even the poorest electron donor, galangin, with the inactivation rate constant of $k = 8.8 \times 10^2 M^{-1} S^{-1}$ (Jovanovic *et al.* 1994), still efficiently scavenged superoxide radical (Jovanovic *et al.* 1998). In this regard, kaempferol and its glycosides are probably efficient scavengers of singlet oxygen and proxyl free radicals as shown in the present study. Although the six major kaempferol glycosides present in soy leaves could not protect LDL from Cu^{2+}-mediated oxidation, they may protect various biomolecules from oxidation and prevent cell death and mutations (Sloley *et al.* 2000 and Asgary *et al.* 1999). Other components in soy leaves may also be beneficial to humans including genistein and its glycosides on cardiovascular system and on cancer prevention (Lichtenstein 1998; Kerry *et al.* 1998; Arorra *et al.* 1998 and Lamartiniere 2000).

The experimental results in this study suggest that the major antioxidant present in soy leaves has not been isolated. Further studies should be carried out to isolate unknown antioxidant(s).

Table 4.2 Composition of the flavonoids and their glycosides present in soy leaves butanol extract.

	Flavonoids (mg/g)
Kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside	52.0 ± 1.2
Kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside	130 ± 2.6
Kaempferol-3-O-digalactopyranoside	56.2 ± 0.4
Kaempferol-3-O-diglucopyranoside	43.5 ± 0.3
Kaempferol-3-O-α-L-rharmnopyranosyl (1→6)- β-D-galactopyranoside	81.5 ± 0.6
Kaempferol-3-O-rutinoside	104.4 ± 1.2
Genistin	6.7 ± 0.1
Malonyl-genistin	12.6 ± 0.1
Genistein	0.0 ± 0.0

Data are expressed as mean ± S.D. of n= 3

Chapter 5

Relaxing effects of soy leaves and its flavonoids

5.1 Introduction

An increased total peripheral resistance is a universal characteristic of hypertension. The increase in the total peripheral resistance is usually due to the hypertensive contraction of the smooth muscles in the blood vessels. Hypertension is dangerous for a number of reasons. First, high arterial pressure makes the ventricles more difficult to eject blood. As a result, the heart must work harder, which can result in pathological changes in heart structure and function, leading to congestive heart failure. Additionally, hypertension increases the risk of heart attack and produces damage to the kidneys, nervous system, and especially the eyes. High pressure may damage cerebral blood vessels, leading to cerebrovascular stroke. Finally, hypertension contributes to the development of atherosclerosis. This may due to the adaptive response to prolonged high blood pressure making the arterial wall becomes thickened.

Vasodilators are the agents that can directly act on the smooth muscle cells and decrease the contractile responses of the smooth muscles in the blood vessels. These vasodilators can either decrease the elevated contractile tone or preventing the increase in contractile response. Since the leading causes of vascular hypertension

are not changed by vasodilators, therefore, vasodilators do not cure hypertension. However, the effect of dilating or preventing constriction of resistance vessels by vasodilators can decrease elevated blood pressure or block the onset of increased vascular tone. As a result, the harmful effects caused by hypertension can be decreased.

5.1.1 Smooth muscle contraction

Smooth muscles are arranged in circular layers around the walls of blood vessles. They contain a great deal of actin and some myosin, which produce a ratio of thin-to-thick filaments of about 16:1. The contraction of smooth muscles is triggered by the increase in Ca^{2+} concentration within the cytoplasm of the smooth muscle cells. Extracellular Ca^{2+} influx into the smooth muscle cell is primarily through voltage-sensitive Ca^{2+} channels in the cell membrane. The opening of these Ca^{2+} channels is graded by the amount of depolarization.

In smooth muscles, Ca^{2+} combines with a protein in the cytoplasm called calmodulin. The calmodulin- Ca^{2+} complex thus formed combines with and activates an enzyme called myosin light chain kinase, which catalyzes the phosphorylation of the myosin cross bridges (Walsh *et al.* 1995). The cross bridges must be phosphorylated before they can bind to actin. The concentration of Ca^{2+} in

the smooth muscle cell cytoplasm determines how many cross bridges will combine with actin, and thus determines the strength of contraction. The contraction of smooth muscle is believed through a sliding filament mechanism similar to that in striated muscle.

5.1.1.1 Sliding filament mechanism

This mechanism is involved in both actin (thin filament) and myosin (thick filament) in smooth muscle cells. The actin filament consists of 300 to 400 contractile protein, G-actin monomers, which are arranged in a double row and twisted to form a helix. The myosin filament of smooth muscle is comprised entirely of the contractile protein, myosin. Myosin is a hexameric molecule consisting of two high molecular weight subunits (heavy chain) and four low molecular weight subunits (light chain). The overall configuration is that of an intertwined coiled tail region with two protruding "cross bridge" and "myosin head" regions. Each myosin head contains an ATP-binding site closely associated with an actin-binding site (Smith *et al.* 1987). The myosin head functions as myosin ATPase, splitting ATP into ADP and P_i. This reaction occurs before the myosin head combines with the actin, and indeed is required for activating the myosin head to attach to the actin. When the myosin head binds to the actin, it undergoes a

conformational change that the ADP and P_i are released and the cross bridge changes their orientation, resulting in a power stroke. This power stroke pulls the actin filaments and causes the actin and myosin filaments to move past one another (Figure 5.1).

5.1.2 Intracellular mechanisms involved in the regulation of smooth muscle contraction

5.1.2.1 Voltage-gated Ca^{2+} channels

Voltage-gated calcium channels are sensitive to changes in the electrical potential across the cell membrane. Upon depolarization, these channels change from resting to activated states within milliseconds and then inactive rapidly. Repolarization is necessary to return to the resting state again (Hockerman *et al.* 1997). The entry of Ca^{2+} into cells through voltage-gated channels is coupled to many cellular responses, such as neurosecretion and muscle contraction. Several types of voltage-gated Ca^{2+} channels have been identified and have been designated L, N, P, Q, R, and T. In smooth muscle, only the L-type Ca^{2+} channel is considered to be a major Ca^{2+} influx pathway (Ganitkevich and Isenberg 1991; Vogalis *et al.* 1991; Kuriyama *et al.*,1995; Knot *et al.* 1996; Hockerman *et al.* 1997). The L-type Ca^{2+} channel with a high sensitivity to several classes of drugs that have been used as

selective L-type Ca^{2+} channel blockers. The three main classes of L-type-selective Ca^{2+} channel blockers currently available for clinical use are the phenylalkylamines, the benz(othi)azepines, and the dihydropyridines. These drugs bind to three separate receptor sites on L-type Ca^{2+} channels, which are allosterically linked (Gould *et al.* 1983; Glossman *et al.* 1984; Hockerman *et al.* 1997).

High K^+-induced depolarization induces a sustained increase in cystolic Ca^{2+}, due to the opening of the voltage-dependent Ca^{2+} channels, and a sustained contraction.

5.1.2.2 Protein kinase C (PKC) mediated smooth muscle contraction

One common denominator in signal transductions involved adenylate cyclase, a transmembrane receptor-tyrosine kinase, phospholipase C, or an ion channel is the eventual regulation of the activity of a protein kinase. Protein kinase can phosphorylate serine or threonine residues of specific target proteins, changing their catalytic activities.

Protein kinase C is one of the protein kinases that is believed to be involved in the regulation of diverse cellular process such as growth, differentiation, metabolism, secretion and smooth muscle contraction.

Five isoenzymes of PKC (α, β, δ, ε and ζ) have been detected in vascular

smooth muscles, but not all of these appear to be expressed in all vascular smooth muscle tissue (Danthuluri. and Deth 1984; Morgrga and Morgan 1984; Khalil *et al.* 1992; Kanashiro and Khalil 1998).

A possible substrate of PKC in the smooth muscle is a specific, and thin filament associated protein, calponin. Calponin is phosphorylated in intact smooth muscle strips in response to carbachol, endothelin-I, phorbol esters, or okadaic acid. Phosphorylation of calponin in vitro by PKC dramatically reduces its affinity for F-actin and alleviates its inhibition of the cross-bridge cycling rate. As a result, it can cause the contraction of smooth muscle (Walsh *et al.* 1996).

Kanashiro and Khalil (1998) suggest that eicosanoid-induced contraction is associated with significant PKC activity that is dependent on Ca^{2+} entry and may involve activation and translocation of the Ca^{2+}-dependent a-PKC isoform. Three subclasses of eicosanoid are postaglandins, leukotrienes and thromobaxanes.

5.1.2.3 Thromboxane A_2 receptor-mediated calcium channel

Thromboxane A_2 (TXA_2) is formed from its precursor, arachidonic acid. It has a six-membered ring containing an ether. It possesses a high affinity for the TXA_2 receptor mediated Ca^{2+}channel and produces the Ca^{2+} influx (Tosun *et al.* 1997). The influx of Ca^{2+} can cause smooth muscle contraction.

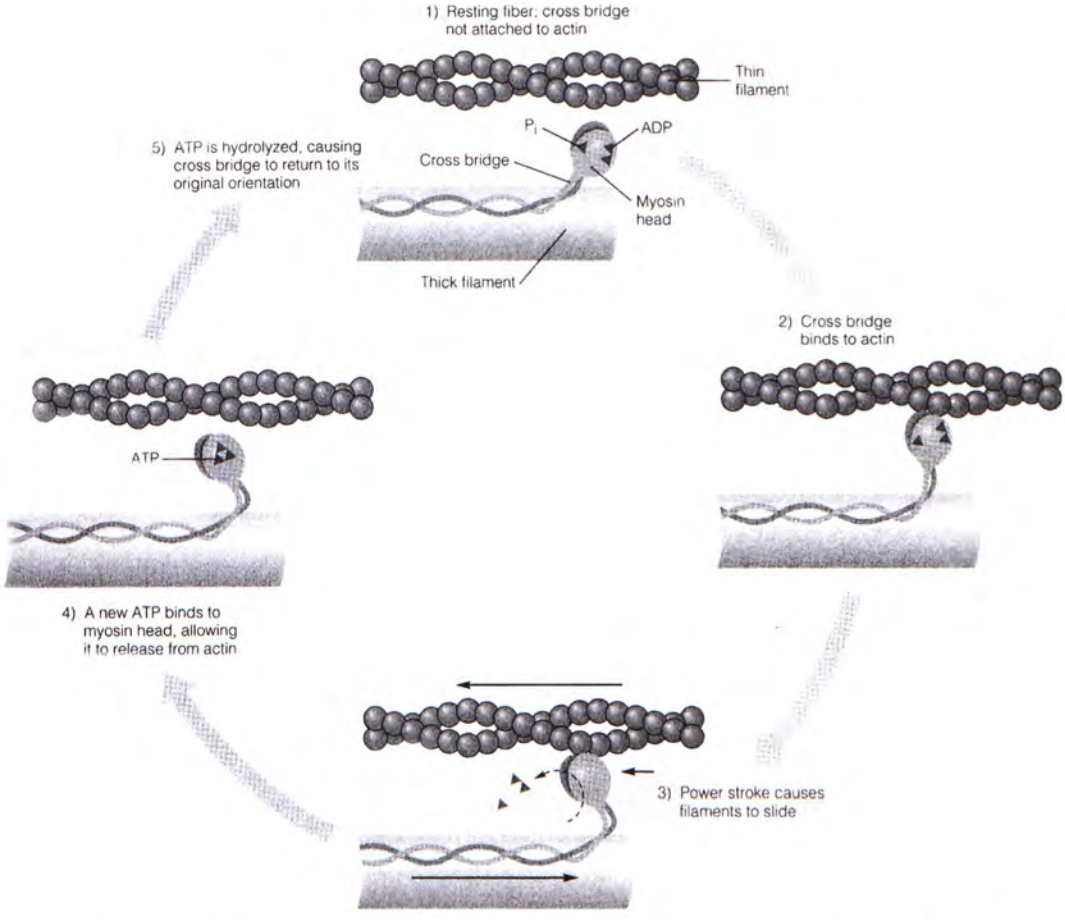


Figure 5.1 The cycle of sliding filaments and muscle contraction (Adapted from Stuart 1996)

5.2 Objectives

The objective of the present study was to examined the effects of soy leaves and its flavonoids on the contractile response to various agonists in rat isolated carotid artery rings.

5.3 Materials and methods

5.3.1 Drugs preparation

The following drugs used include phenylephrine hydrochloride, acetylcholine hydrochloride, 9,11-dideoxy-11$_{\alpha}$,9$_{\alpha}$-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619), [5Z,9$_{\alpha}$,11$_{\alpha}$,13E,15S]-9,11,15-trihydroxyprosta-5,13-dienoic acid ($PGF_{2\alpha}$), phorbol 12-myristate 13-acetate (PMA), glibenclamide, nifedipine, staurosporine, paraverine and kaempferol (Sigma, St. Louis, MO, USA). Soy leaves butanol extract and kaempferol glycosides were purified as described in **Chapter 2** part ***2.3.1.1*** and ***2.3.1.2.*** Glibenclamide, PMA, staurosporine and nifedipine were dissolved in dimethyl sulfoxide (DMSO) and others in distilled water. DMSO at 0.2-0.6% (v/v) did not affect the sustained contraction induced by U46619.

5.3.2 Vessel preparation

After the approval from Animal Ethical Committee of Chinese University of Hong Kong was obtained, male Sprague-Dawley rats (250-300 g) were killed by cervical dislocation. The common carotid artery from both sides was dissected free from surrounding tissues and cut into rings of 3 mm in length. The preparation was then transferred into 10 ml organ baths containing Krebs solution bubbled with a mixture of 95% O_2 plus 5% CO_2. Each ring was mounted between two L-shaped stainless steel

hooks. One hook was mounted at the bottom of the bath while the other was connected to FT03 force-displacement transducer (Grass Instruments Co). Krebs solution contained (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$,1.2 KH_2PO_4, 11 d-glucose. A passive tone of 5 mN was applied to all artery rings. All experiments were performed at 37 °C. In some arterial rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the artery back and forth several times with plastic tubing. The tissues were allowed to equilibrate for 90 minutes, during which the bath solution was replaced with pre-warmed and oxygenated Krebs solution every 15 minutes. Endothelium integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response to 1 μM acetylcholine under the contraction induced by 10μM phenylephrine at the start of each experiment. Then the rings were rinsed with pre-warmed and oxygenated Krebs solution several times until the basal level of tension was restored. The rings were allowed to equilibrate for additional 60 minutes. Each experiment was performed on the rings prepared from different rats.

5.3.3 Contraction experiments

5.3.3.1 Relaxant responses of soy leaves butanol extract on the contraction induced by different constrictors

In the first set of experiments, a sustained vessel tone was induced by three constrictors, 30 nM U46619, 10 μM phenylephrine and 50 mM external K^+, soy leaves butanol extract was added cumulatively to the bathing solution. These experiments were also repeated in the endothelium-denuded artery rings to examine the possible involvement of endothelium in extract-induced relaxant response. The effect of extract was also tested on the contractile response to 1 μM PMA in Ca^{2+}-free Krebs solution containing 0.5 mM Na_2-EGTA. In control experiments, Glibenclamide (an antagonist of prostaglandins) was used to inhibit U46619-induced contraction; nifedipine (L-type voltage-gated Ca^{2+} channel blocker) used to inhibit the high K^+ response; and staurosporine (a protein kinase C inhibitor) used to inhibit PMA-induced contraction.

In the experiments using high K^+ solution, Na^+ was replaced with an equimolar amount of K^+ to maintain the ionic strength.

5.3.3.2 Relaxant responses of soy leaves butanol extract on U46619 and PGF2α-induced contraction

In the second series of experiments, the endothelium-intact ring was contracted by U46619 (0.03-300 nM) or $PGF_{2\alpha}$ (0.03- 3 μM) to construct the first concentration-contraction curve. Once the maximum contraction was obtained, the ring was washed off the constrictor with the pre-warmed Krebs solution several times until the basal tone was reestablished. The ring was then incubated with the extract of different doses for 30 min and another concentration-response curve to constrictor was repeated.

5.3.3.3 Relaxant responses of genistein, genistin and the kaempferol glycosides on U46619-induced contraction

In the last series of experiments, the effects of purified individual kaempferol glycosides, a mixture of kaempferol glycosides, genistein and genistin were examined in U46619-preconstricted artery rings.

5.3.4 Statistics

The relaxant effect of the extract and other relaxants was expressed as percentage relaxation of the agonist-constricted arterial rings. When drug was given in g/L, IC_{50} was calculated as the drug concentration inducing 50% of the maximum inhibition and this value was presented together with 95% confidence intervals. When constrictor was given in molar per liter, pD_2 values were calculated as negative log concentration

that caused 50% of the maximum contraction. Data were presented as means ± S.E.M. of *n* experiments. Statistical significance was analyzed by Student's *t* test or by one way ANOVA followed by Newman-Keuls test when more than two treatments were compared. A p value less than 0.05 was considered significant.

5.4 Results

5.4.1 Effect of soy leaves butanol extract

Traces in Figure 5.2 showed the effect of soy leaves butanol extract on the isolated rat carotid artery rings preconstricted by 30 nM U46619 (Figure 5.2a), 10 μM phenylephrine (Figure 5.2b) or 50 mM extracellular K^+ (Figure 5.2c). In U46619-preconstrcted endothelium-intact rings, soy leaves butanol extract showed a dose-dependent relaxation with an IC_{50} of 0.19 (0.15-0.23) g/L (n= 5, Figure 5.3). The complete relaxation was achieved at a dose of 1 g/L (n= 5). The extract only induced partial relaxation of rings preconstricted by phenylephrine and 31 ± 5.6% relaxation was obtained with the extract at 1 g/L (n= 4, Figure 5.3). In contrast, the extract had no effect on 50 mM K^+-contracted rings (n= 4, Figure 5.3), but nifedipine at 100 nM completely inhibited the high K^+ response (n= 4). Besides, the extract did not alter contraction induced by active phorbol ester, PMA (1 μM) in Ca^{2+}-free Krebs solution (n= 4, Figure 5.3).

5.4.2 Role of endothelium in extract-induced relaxation

Figure 5.4a showed that the extract induced dose-dependent relaxation in U46619-preconstricted rings in both endothelium-intact and -denuded rings with the same potency (IC_{50}: 0.19 (0.15-0.23) g/L, n= 5 with endothelium and 0.16 (0.12-0.20)

g/L, n= 4 without endothelium, p >0.05). Similarly, the endothelium was not involved in the partial relaxant response to the extract in phenylephrine-preconstricted rings (n= 4-5, Figure 5.4b). The extract did not relax rings contracted by 50 mM K^+ regardless the presence or absence of the functional endothelium (n= 4, Figure 5.4c). Staurosporine (100 nM), an inhibitor of protein kinase C, completely abolished PMA-induced contraction.

5.4.3 Effect of the soy leaves butanol extract on contractile response to prostaglandins

Since the extract was significantly more potent to relax rings contracted by U46619 than phenylephrine, it was worthwhile testing whether the yet-to-be-identified active components in the extract may act as pharmacological antagonist(s) of prostaglandin-induced vasoconstriction. Figure 5.5 showed that pretreatment with the extract caused approximately parallel rightward shift of the concentration-response curve for U46619-induced relaxation and this effect was dose-dependent with slight suppression of the maximum relaxation. The pD_2 values were 8.04 ± 0.02 in control; 7.62 ± 0.02 in 0.03 g/L extract, 7.11 ± 0.02 in 0.3 g/L extract, and 7.00 ± 0.04 in 1.0 g/L extract (n= 4-5, $p < 0.05$ compared with the control value, Figure 5.5a). The extract also reduced the concentration-dependent contractile

responses to $PGF_{2\alpha}$ (Figure 5.5b). Traces for the concentration-response curves of U46619 were shown in Figure 5.6. In control experiments, glibenclamide was used to relax U46619-contracted rings as an antagonist of prostaglandin-induced vascular response (n= 4, pD_2 values = 6.57 ± 0.08).

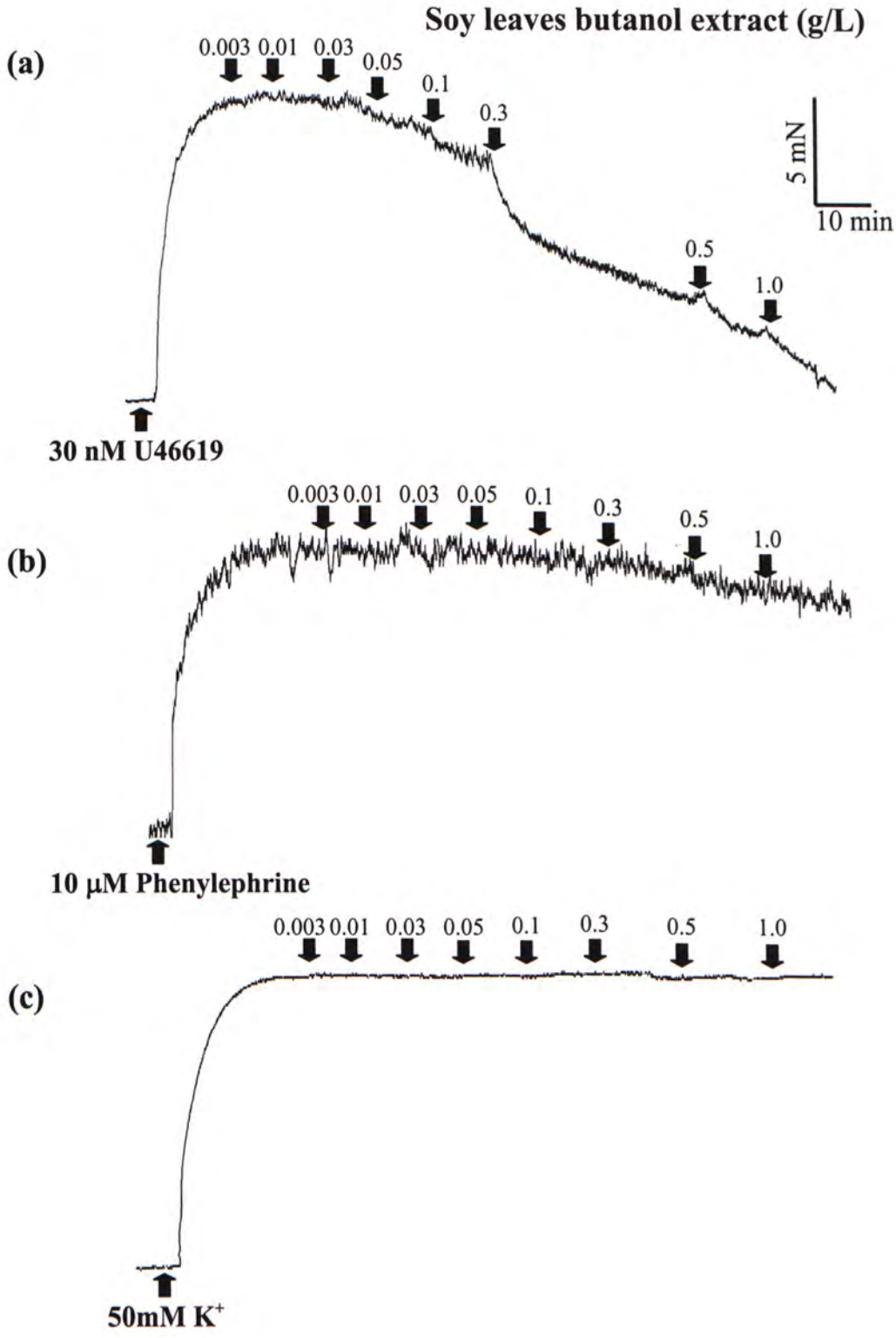


Figure 5.2 The representative recordings showing the vascular effect of soy leaves butanol extract on the isolated rat carotid arteries with endothelium preconstricted by 30 nM U46619 (a), 10 μM phenylephrine (b), and 50 mM K^+ (c). The calibration bars apply to all traces.

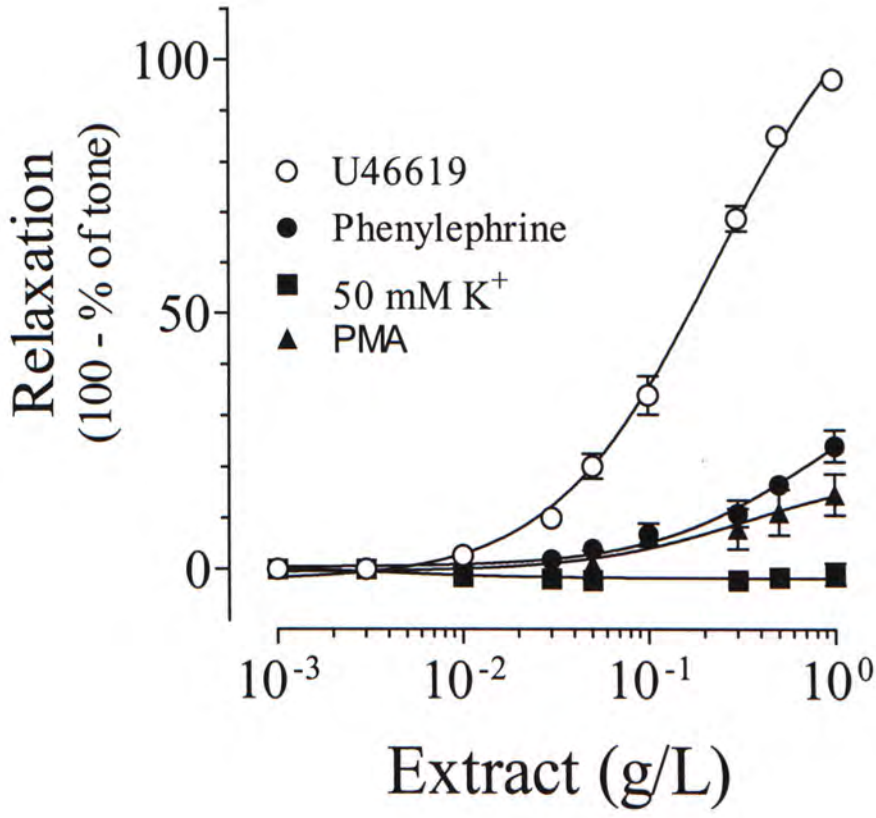


Figure 5.3 The concentration-response curve for the extract-induced effect in endothelium-intact rings preconstricted by U46619 (○, n= 5), phenylephrine (●, n= 5), 50 mM K^+(■, n= 4) and PMA (▲, n= 4). Data are expressed as means ± S.E.M. of n experiments.

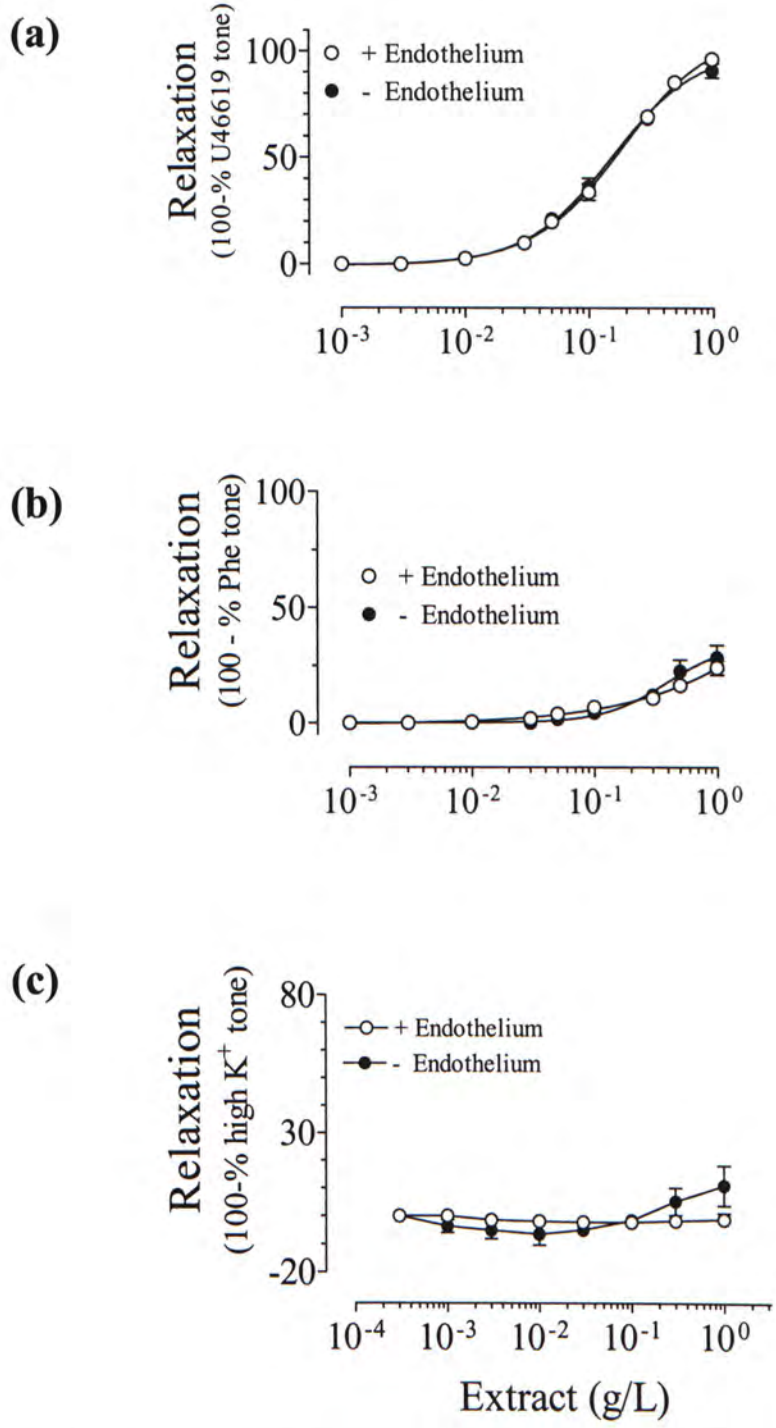


Figure 5.4 Effect of soy leaves butanol extract in rat carotid arteries pre-constricted by 30 nM U46619 (**a**), 50 mM K^+ (**b**) and 10 μM phenylephrine (**c**) in the presence (○, n= 4-5) and absence of endothelium (●, n= 5-7). Data are expressed as means ± S.E.M. of n experiments.

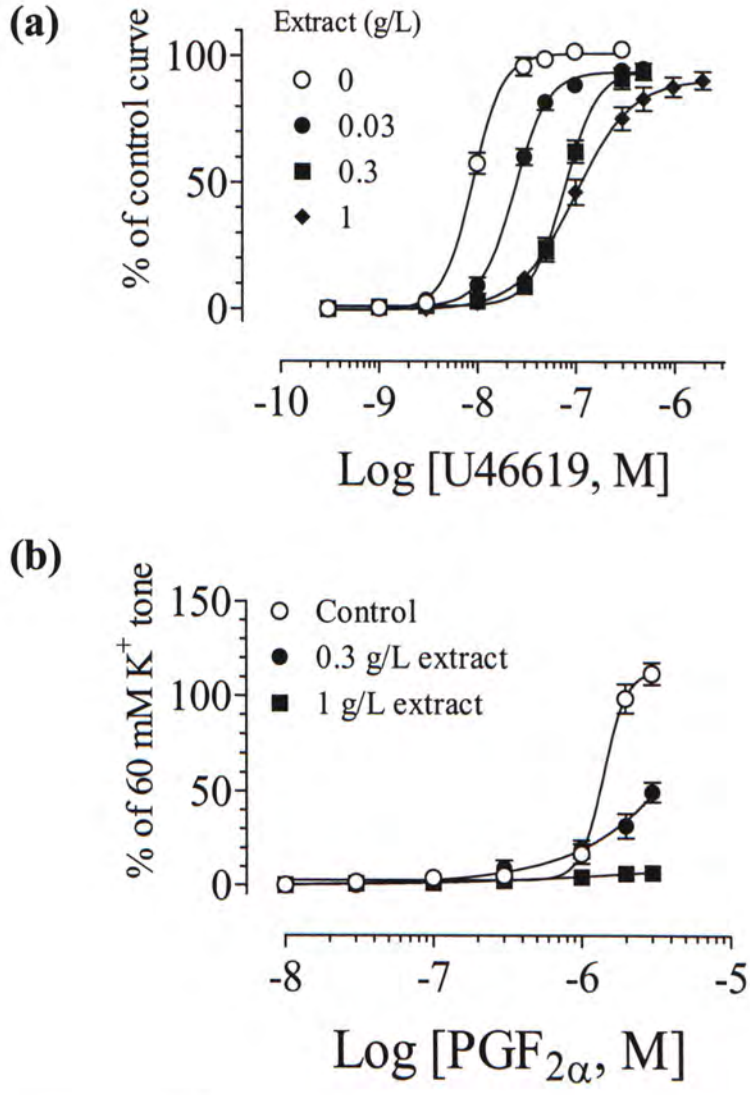


Figure 5.5 (**a**) Concentration-dependent relaxing effect of soy leaves butanol extract on U46619-contracted arterial rings with endothelium (○, in control; ●, 0.03 g/L; ■, 0.3 g/L; ◆, 1 g/L butanol extract, n= 6 in each case). (**b**) Concentration-dependent relaxing effect of soy leaves butanol extract on $PGF_{2\alpha}$-contracted arterial rings with endothelium (○, in control; ●, 0.3 g/L; ■, 1 g/L butanol extract, n= 4 in each case). The rings were incubated with extract at different doses for 30 min before the second concentration-response curve was repeated. The contractile responses were expressed as percentages of the maximum contraction in the first (control) concentration-response curve. Data are expressed as means ± S.E.M. of n experiments.

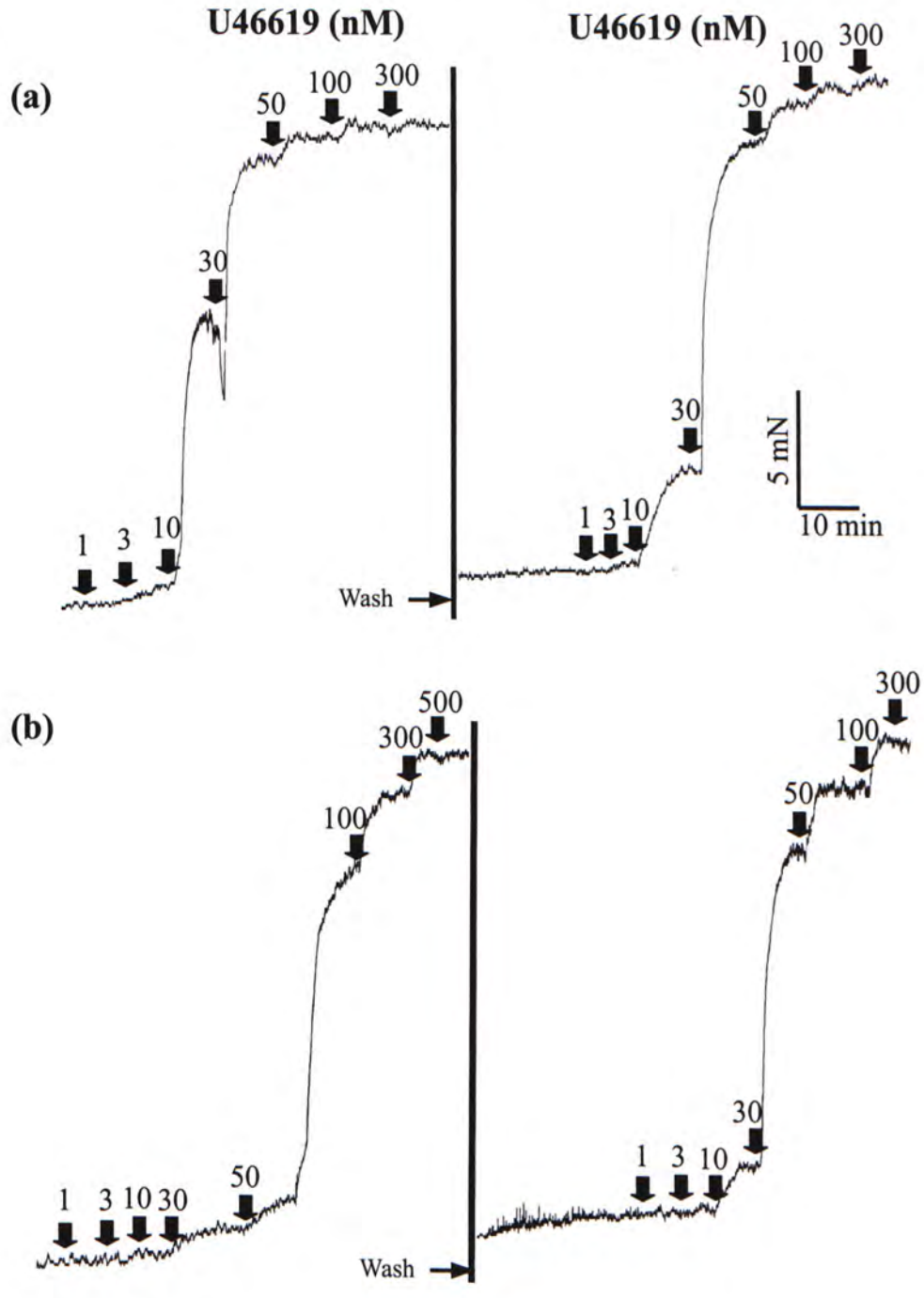


Figure 5.6 (a and b). Traces showing the contractile response of U46619 in the absence (a) and in the presence of 0.03 g/L soy leaves butanol extract (b). The tissues were incubated with the drugs for 30 minutes before the second dose response curves of U46619 (1-500 nM) were constructed. Calibration bars apply to the traces.

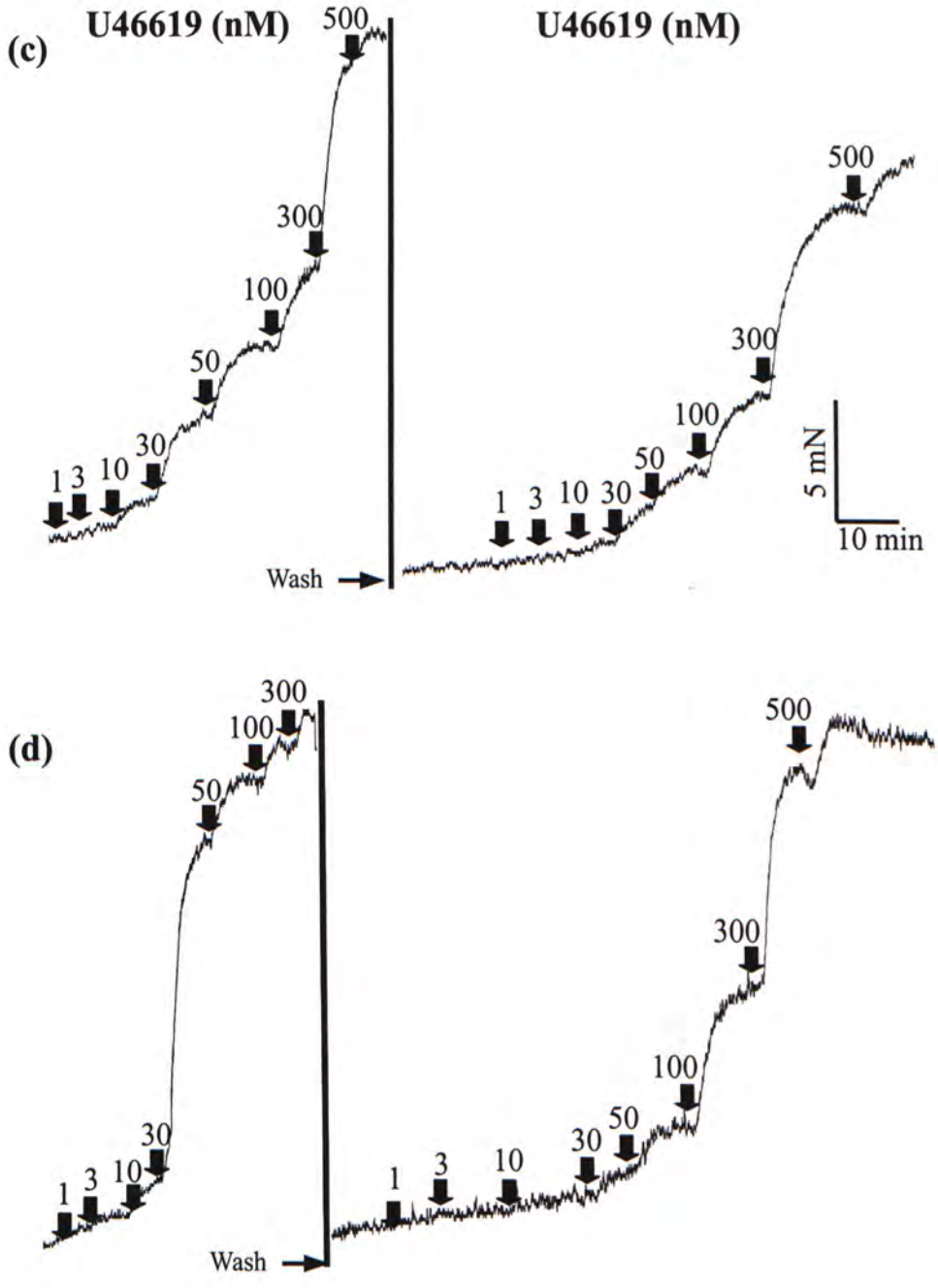


Figure 5.6 (c and d). Traces showing the contractile response of U46619, in the presence of 0.3 g/L (c) and 1.0 g/L soy leaves butanol extract (d). The tissues were incubated with the drugs for 30 minutes before the second dose response curves of U46619 (1-500 nM) were constructed. Calibration bars apply to the traces.

5.4.4 Effects of kaempferol glycosides and kaempferol

The six kaempferol glycosides from the butanol extract, which accounted for 47.8% (w/w) in it (Table 5.1). The HPLC chromatogram was shown in Figure 5.7. In order to search for the responsible components for the extract-induced relaxation, the individual glycosides were tested in U46619-preconstrcited endothelium-intact rings. Figure 5.8 showed that none of the six kaempferol glycosides affected the vessel tone (n= 4-5). In contrast, kaempferol without sugar molecules produced concentration-dependent relaxation (IC_{50}: 0.40 (0.35 to 0.45) mg/L, n= 4, Figure 5.9). In order to examine further whether a mixture of kaempferol glycosides may have a synergestic effect on vessel relaxation, a mixture of kaempferol glycosides was prepared according to their relative composition by weight in the extract. This mixture did not change the contractile response to U46619 (n= 5, Figure 5.9).

5.4.5 Effects of genistein and genistin

The extract also contained genistin and malonyl-genistin, which accounted for 0.67% and 1.26% of the butanol extract in weight, respectively. A minute amount of genistein was also present in the extract. Both agents relaxed the endothelium-intact rings in a dose-dependent manner with genistein being more potent than genistin (pD_2 values were 5.74 ± 0.03 (n= 4) in genistein, 4.09 ± 0.19 (n= 4) in

genistin (Figure 5.10). Genistein at 10 μM produced near maximum relaxation (90.3 ± 1.9%), while this concentration of genistin had no effect. Genistin at 300 μM produced 55.9 ± 7.6% relaxation (n= 5, Figure 5.10).

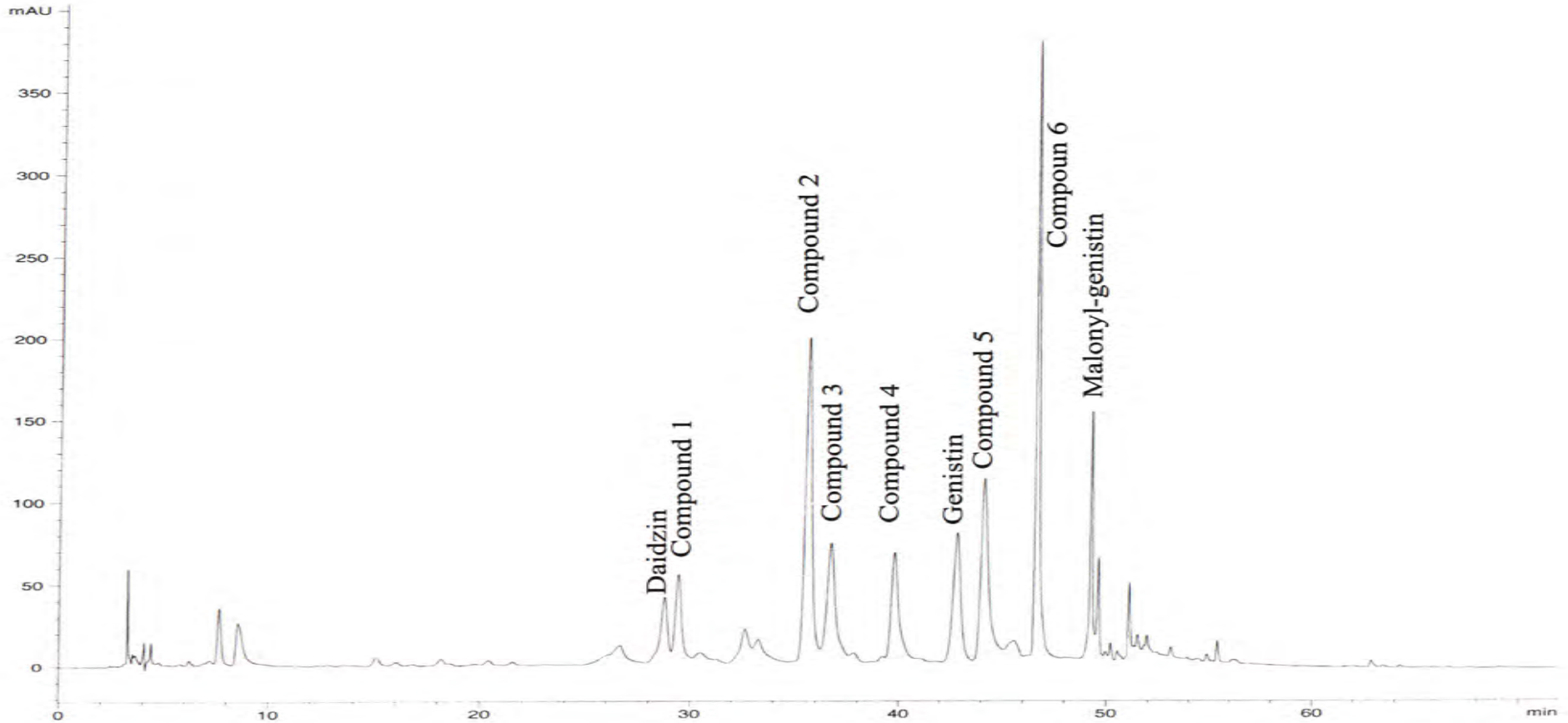


Figure 5.7 HPLC chromatogram of soy leaves butanol extract. Compound 1, kaempferol-3-O-α-L-rhamnopyranosyl(1→2)β-D-glucopyranosyl (1→6) -β-D-galactopyranoside; Compound 2, kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside; Compound 3, kaempferol-3-O-digalactopyranoside; Compound 4, kaempferol-3-O-diglucopyranoside; Compound 5, kaempferol-3-O-α-L-rharmnopyranosyl (1→6)- β-D-galactopyranoside; Compound 6, kaempferol-3-O-rutinoside.

Table 5.1 Percentage of flavonoids and flavonoid glycosides in soy leaves butanol extract

	% in soy leaves butanol extract (w/w)
Kaempferol-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-galactopyranoside	5.2 ± 0.1
Kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside	14.0 ± 0.3
Kaempferol-3-O-digalactopyranoside	5.6 ± 0.1
Kaempferol-3-O-diglucopyranoside	4.4 ± 0.1
Kaempferol-3-O-α-L-rharmnopyranosyl (1→6)- β-D-galactopyranoside	8.2 ± 0.1
Kaempferol-3-O-rutinoside	10.4 ± 0.1
Genistin	0.7 ± 0.1
Malonyl-genistin	1.3 ± 0.1
Genistein	0.0 ± 0.0

Data are expressed as mean ± S.D. (n= 3)

Kaempferol glycosides in soy leaves butanol extract (w/w) = 47.8%

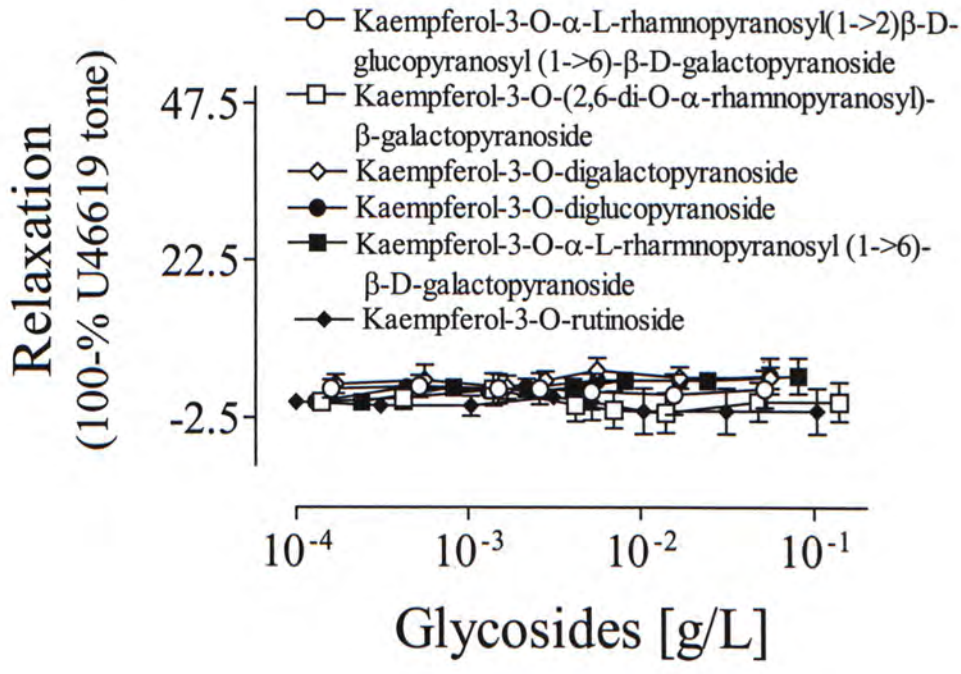


Figure 5.8 Effects of six kaempferol glycosides on 30 nM U46619-contracted endothelium-intact arterial rings (n= 4 in each case). Data are means ± S.E.M. of n experiments.

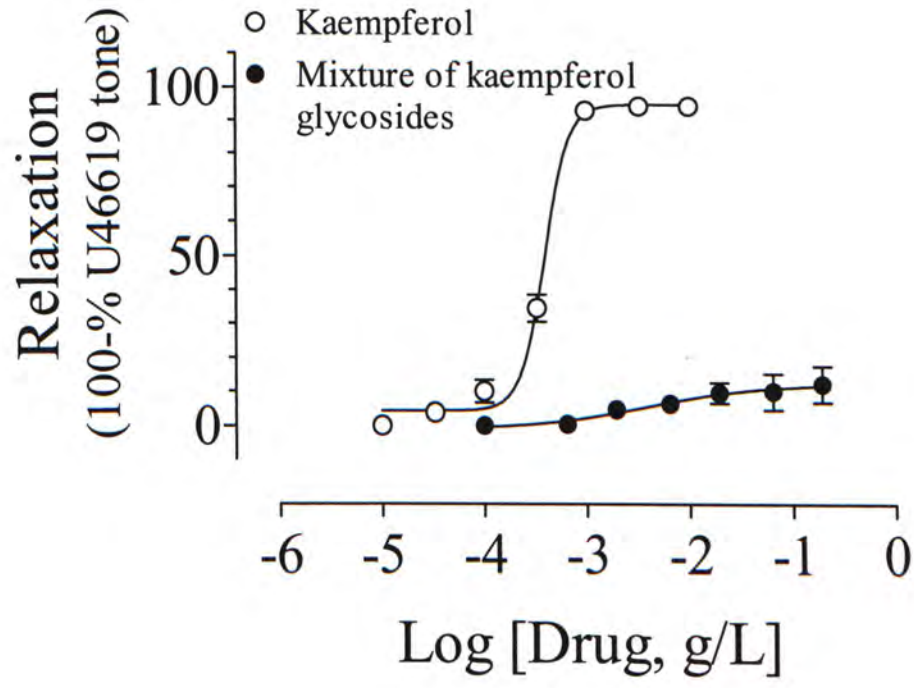


Figure 5.9 Effect of kaempferol (○, n= 4) and a mixture of kaempferol glycosides on U46619-contarcted rings (●, n= 4). Data are means ± S.E.M. of n experiments.

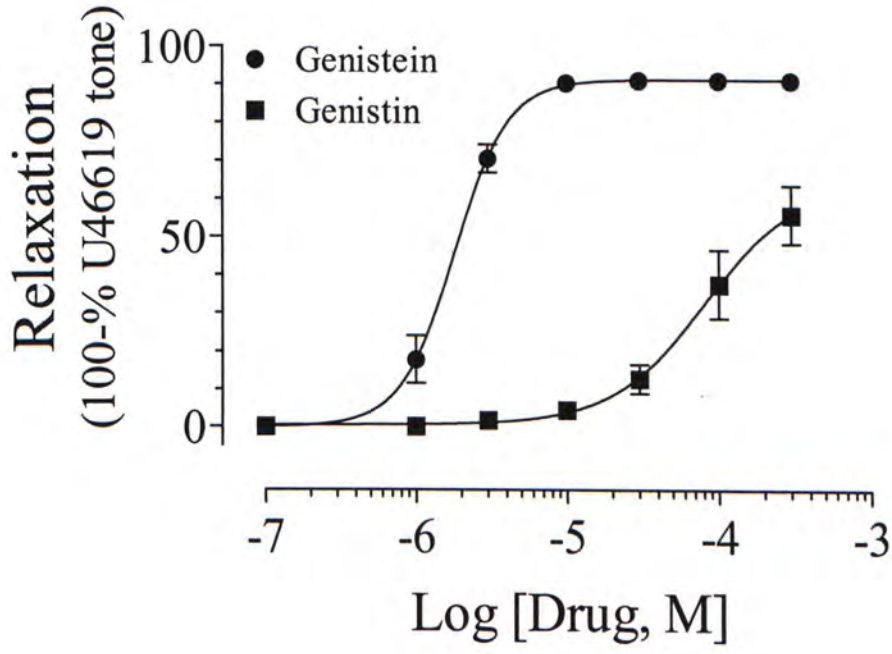


Figure 5.10 Effects of genistein and genistin in rat carotid arteries pre-constricted by 30 nM U46619 in the presence of endothelium (●, n= 4 in genistein and ■, n= 5 in genistin). Data are means ± S.E.M. of n experiments.

5.5 Discussion

The present study was the first attempt to examine the vascular effects of soy leaves extract and its bioactive ingredients. The present results showed that soy leaves butanol extract dose-dependently relaxed the isolated rat carotid arteries. The relaxant effect was much greater in rings preconstricted by U46619, a potent thromboxane A_2 receptor agonist, than by phenylephrine, a selective α-adrenoceptor agonist. It is clear that some unknown components contained in the extract possess vasorelaxant property. The extract-induced vasorelaxation was independent of the presence of the functional endothelium since endothelium removal did not affect the relaxing potency, indicating that endothelium-derived vasoactive factors, either relaxing or constricting, were not involved. U46619 and phenylephrine contract blood vessels via similar mechanisms, depending on the presence of extracellular calcium ions. A substantial amount of agoinst-induced Ca^{2+} influx occured via voltage-gated Ca^{2+} channels since nifedipine, a Ca^{2+} channel antagonist produced marked inhibition of agonist-induced vasoconstriction. Protein kinase C activated by both diaceylglycerol and intracellular Ca^{2+} is reported to facilitate Ca^{2+} entry by promoting voltage-gated Ca^{2+} channel activity. However, the present results rule out the possible inhibitory effects of the extract on contractile response induced by Ca^{2+} influx through voltage-gated Ca^{2+} channels or protein kinase C activation.

Firstly, the extract had no effect on the contraction induced by 50 mM K^+. It is generally believed that one of the major consequences of raising extracellular K^+ is to open voltage-gated Ca^{2+} channels by membrane depolarization of the vascular smooth muscle. In contrast, nifedipine completely inhibited the high K^+-evoked contraction. This indicates that the extract does not contain any components that may function as antagonists of Ca^{2+} channels. In endothelium-denuded rings bathed in Ca^{2+}-free medium, a slowly developed contractile response can be evoked by PMA, exogenous activator of protein kinase C, suggesting that protein kinase C stimulation may interact with contractile filaments at the resting cytosolic Ca^{2+} level (Huang 1996). The extract did not alter the PMA-induced contraction, implying that inhibition of protein kinase C-mediated pathway was not the mechanism responsible for extract-induced relaxation. In control experiments, the protein kinase C inhibitor staurosporine totally suppressed PMA-induced response.

The present results showed that the extract was much more effective in relaxing rings preconstricted by U46619 than phenylephrine, suggesting the possible antagonistic action of vasoconstricting prostaglandins. Indeed, the extract at 0.03 and 0.3 g/L caused parallel right ward shift of the concentration-response curve for U46619-induced contraction while the extract at 1 g/L slightly reduced U46619-induced maximum contraction. In addition, the extract also dose-dependently

reduced contractile response to $PGF_{2\alpha}$, another member of vasoconstricting prostaglandins. These data suggest that some unidentified components of the extract that may act as prostaglandin antagonists, which needs to be further investigated.

Another objective of the present study was to identify the bioactive components of the extract. In this regard, six kaempferol glycosides were isolated. It was found that these six glycosides accounted for 48% of total extract weight. However, none of these six kaempferol glycosides affected U46619-induced contraction. Besides, when a mixture of six glycosides was prepared according to their relative percentage composition in the extract, no effect could be observed. These results clearly showed that kaempferol glycosides were inactive in relaxing the rat carotid arteries. In contrast, kaempferol caused concentration-dependent relaxation. If kaempferol glycosides can be converted to kaempferol during intestinal absorption by losing their sugar moieties, the latter should be able to possess vasorelaxant effect. The extract also contained a small amount of genistin (1.26% in weight), but the amount of genistein was too little to be quantified by HPLC analysis. Genistin caused a dose-dependent relaxation. However, the concentration of genistin at 35.11 mg/L (calculated from the pD_2 value of genistin) caused 50% relaxation in U46619-induced contraction was much higher than that 1.27 mg/L contained in 0.19

g/L extract that cause 50% relaxation in U46619-induced contraction, indicating that genistin was not the main ingredient accounting for the observed relaxation induced by the extract. The present study was also to examine the effect of genistein, which was purchased from Sigma with a purity over 98%. Genistein was a potent relaxant against U46619-induced contraction. Even if genistein was formed from genistin, this would contribute little to the extract-induced relaxation.

Taken together, soy leaves extract relaxed the isolated rat carotid arteries pre-constricted by thromboxane A_2 and prostaglandin $F_{2\alpha}$. The extract also relaxed the arterial rings contracted by phenylephrine albeit to a much less degree. The extract (0.03 and 0.3 g/L) caused relaxation indicating that some component(s) in soy leaves extract could act as prostaglandin antagonist(s). Lack of effect on contractile response to raised extracellular K^+ which activates Ca^{2+} channels, or active phorbol ester which activates protein kinase C, indicated that the extract was not comprised of any components which may inhibit either voltage-gated Ca^{2+} channels or protein kinase C-dependent cellular mechanism. Kaempferol glycosides account for about 48% in total extract weight, however, none of these glycosides relaxed preconstricted rings, showing that kaempferol glycosides were devoid of vasorelaxant activity. These kaempferol glycosides may become kaempferol by the bacteria in the intestine (Kuhnau 1976; Hollman and Katan 1999) which then absorbed into the blood and

relax blood vessels. Lack of a relaxing effect of genistin, although it was contained in the extract, on the contraction at doses that fall within the effective dose range of the extract. This suggested that genistin was not a responsible ingredient of the extract at doses used for vasorelaxation. Further experiments are needed to identify the active components that actually account for the vasorelaxant effect of soy leaves extract and to examine their vascular action. There is limited information in literature regarding the bioactive components of soy leaves even though soy leaves are abundantly available. By comparison with soybean, phytoestrogenic agents such as genistein is present only in a minute amount in soy leaves. On the other hand, soy leaves contains kaempferol glycosides but not in soybean. Therefore, soy leaves may be able to use as an effective vasorelaxant.

Chapter 6

Effect of soy leaves on mammary tumor

6.1 Introduction

Breast cancer is the most common cancer and the second most frequent cause of cancer death in women (Parker *et al.* 1997). Several factors such as genetic, ovarian hormonal level, nutrition and life style may play a significant role in the development of breast cancer. Epidemiological data suggest that higher intakes of soy protein are associated with lower rates of breast cancer (Lee *et al.* 1991; Messina *et al.* 1994; Wu *et al.* 1998). Some studies support that isoflavones are the major component in soybean that are responsible for this cancer preventing ability (Barnes 1995; Peterson and Barnes 1996).

6.1.1 Carcinogenesis

Carcinogenesis, the process of cancer, consists of three stages: initiation, promotion and progression. However, the real situation is more complex and the stages less distinct.

6.1.1.1 Initiation

It is believed that proto-oncogenes become oncogene through mutation, which occurs when a gene is altered in some way. Most of the time, mutations are harmless because they occur in genes that have little effect on cell function. But when the mutation occurs in the "right" place in a proto-oncogene, the result is the formation of an oncogene.

Sometimes, mutations occur spontaneously, but more often, they are due to an outside influence. Something that causes a mutation is called a mutagen. Mutagens that cause cancer are called carcinogens. Examples of carcinogens are some pesticides and herbicides and certain industrial chemicals as well as asbestos and many chemicals in tobacco. X-rays in a high dose and some viruses cause mutation that can result in cancer.

6.1.1.2 Promotion

Initiation involves a permanent and irreversible change in the DNA. Once the change has occurred and the cell has divided, the message to repeat the process is passed on from one generation of cells to the next. Initiation happens very quickly, but most initiated cells probably remain dormant, or inactive, until acted on by

promoters. Without the action of promoters, initiated cells will not progress to a disease stage.

Promoting agents are not carcinogenic in themselves, that is, they cannot cause cancer in non-initiated cells. Promoters induce initiated cells to divide. Initiated cells can grow in number more easily than normal cells. The period between initiation and the appearance of tumors is one of the least understood aspects of tumor development. This period is called the latency period. The latency period can be more than twenty years before tumor develop.

6.1.1.3 Progression

The final stage of carcinogenesis is progression. At this point, the developing tumor is beginning to acquire traits that allow it to grow in size and to invade and spread to other tissues. The spreading of cancer cells to other parts of the body is metastasis. One very important part of this final stage is angiogenesis, the development of new blood vessels. For tumors to grow in size, they need to create their own blood vessel to receive the oxygen and nutrients that all cells need for survival.

6.2 Objective

Soy leaves, not only contains genistein but also its glycosides. Another kind of flavonoid glycosides, kaempferol glycosides, which may be beneficial in cancer prevention (Dimas *et al.* 2000), is also present in it. In the present study, the anticarcinogenic effect of soy leaves on mammary tumor was investigated using female Sprague-Dawley rats as an animal model.

6.3 Materials and methods

6.3.1 Animal

Female Sprague-Dawley rats were housed (3- 4 rats per cage) in an animal room at 25 °C with 12:12-h light-dark cycles. Fresh semi-synthetic diets were given daily, and uneaten food was discarded. Food intake was measured daily and body weight was recorded twice a week. The rats were allowed access to food and fluid *ad libitum.*

All the rats were gavaged with dimethylbenz[a]anthracene (DMBA) (Sigma Chemical, St. Louis, MO, USA), a carcinogen, at the proestrus or estrus phases, in order to diminish the influence of ovarian hormonal level. The amount of DMBA gavaged was according to their body weights (80 mg/kg).

Once the DMBA were gavaged, all the rats (125- 140 g) were randomly divided into 2 groups (n= 20). They were fed a semi-synthetic diet, with or without supplementation of soy leaves powder (SLP). The method described by Appelt and Reicks (1999) was modified and used to prepare the semi-synthetic diet. For the control group, diet was prepared by mixing the powdered ingredients (casein, 200 g; corn oil, 70 g; starch, 509 g; sucrose, 100 g; cellulose, 50 g; mineral mix, 35 g; vitamin mix, 10 g; DL-methionine, 3 g; and choline bitartrate, 3 g) with 200 mL gelatin solution (100g/L). For the SLP group, 3% by weight soy leaves powder

(SLP) was mixed with the control diet before setting them in gelatin. Once the gelatin had set, the diet was cut into approximately 20 g cubic portions and stored frozen (-20 °C).

The percentage dietary fiber content in the control and SLP groups were 4.6 ± 0.2 and 5.6 ± 0.3 respectively (the total dietary fiber content was determined according to the official method described in AOAC 991.43). All the rats were checked weekly to monitor their body weights and palpable tumors.

6.3.2 Determination of estrus cycle

During the reproductive cycle of the mammalian female, the secretion of estrogens and progesterone varies with the phases of the ovarian cycle (Lu *et al.* 1979). The estrogen levels are low after ovulation (estrus phase) and remain at low levels until diestrus when a new set of ovarian follicles begins to mature. By the end of the diestrus phase, serum estrogen levels are significantly elevated and continue to rise until a peak is reached just prior to ovulation (proesturs phase) (Shin and Lee 1978). This in turn produces characteristic changes in the epithelial lining, and in the secretory and muscular activities of the female reproductive tract. In general, the effect of estrogens is to induce vaginal cornification and progesterone stimulates mucification.

A small amount of saline (0.9% NaCl) was flushed into and out of the vagina for 2 to 3 times by using a pasteur pipette. The washing was then spread onto a glass slide and dried in air. Then, it was fixed in methanol and air dried completely. The slide was dipped into 0.1% methylene blue solution for 3 minutes. The excess methylene blue was washed away by tap water. Finally the slide was dried and mounted.

The four phases in the estrus cycle are proestrus, estrus, metestrus and diestrus. In the proestrus and estrus phases, the vaginal smear consisted of mainly nucleated polygonal epithelial cells and cornified squamous epithelial cells respectively. However, in the metestrus and diestrus, the vaginal smear consisted of mainly leucocytes. Figure 6.1a and b showed the vaginal smears in proestrus and diestrus phases respectively.

6.3.3 Statistics

Chi-square test was used for statistical evaluation of differences for the incidence rate and number of tumors between groups at the same week (SigmaStat version 2.01, SigmaStat Advisory Statistical Software, MO, USA).

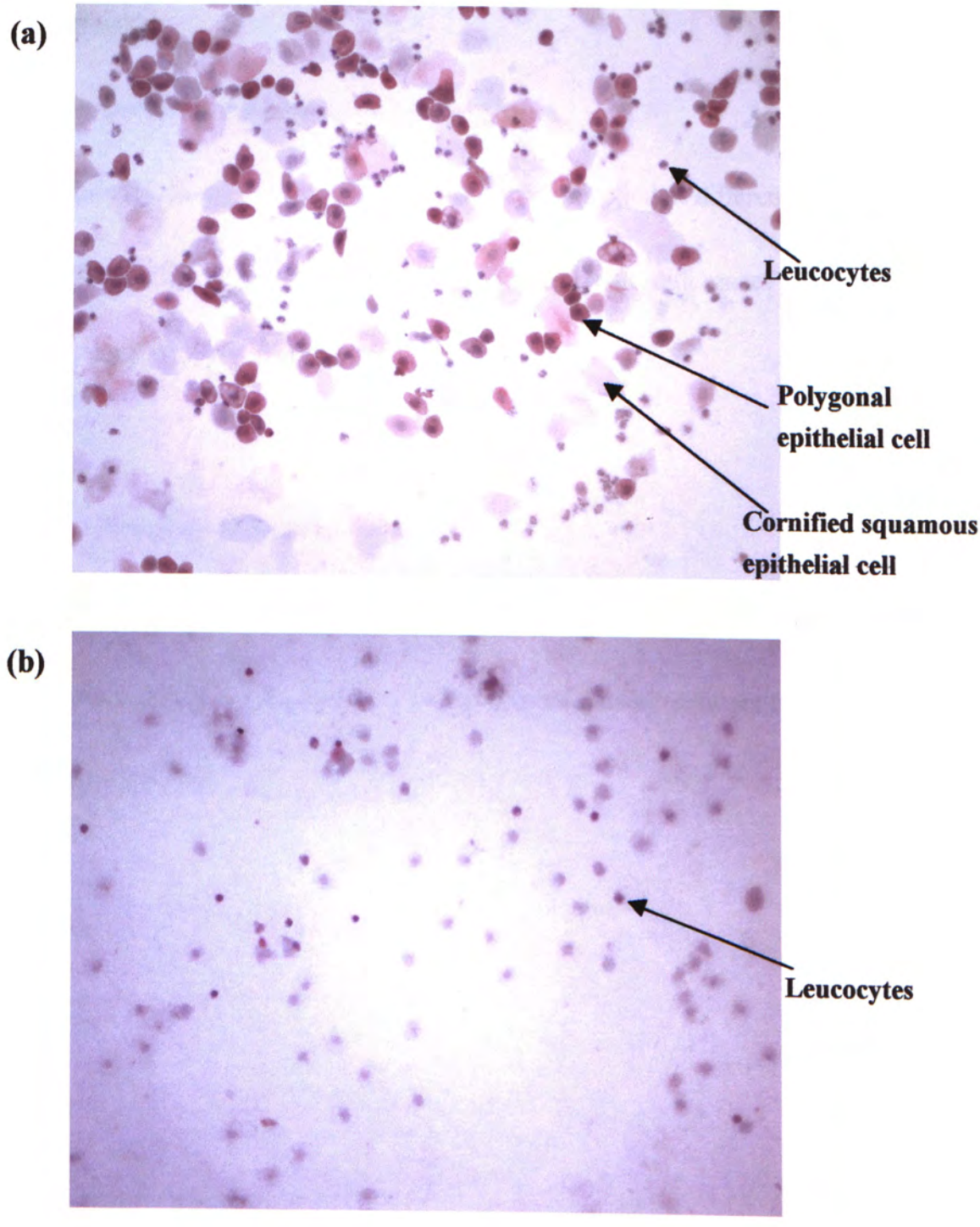


Figure 6.1 Vaginal smears of the rats in proestrus (a) and diestrus (b) phases.

6.4 Results

6.4.1 Incident rate of tumor induction

Figure 6.2 showed the incident rate of tumor induction at different time points during the entire experimental period. No significant differences were found between the two groups at different time points.

6.4.2 Number of tumor induced

Figure 6.3 showed the cumulative number of tumors induced during the entire experimental period. No significant differences were found between two groups at different time points.

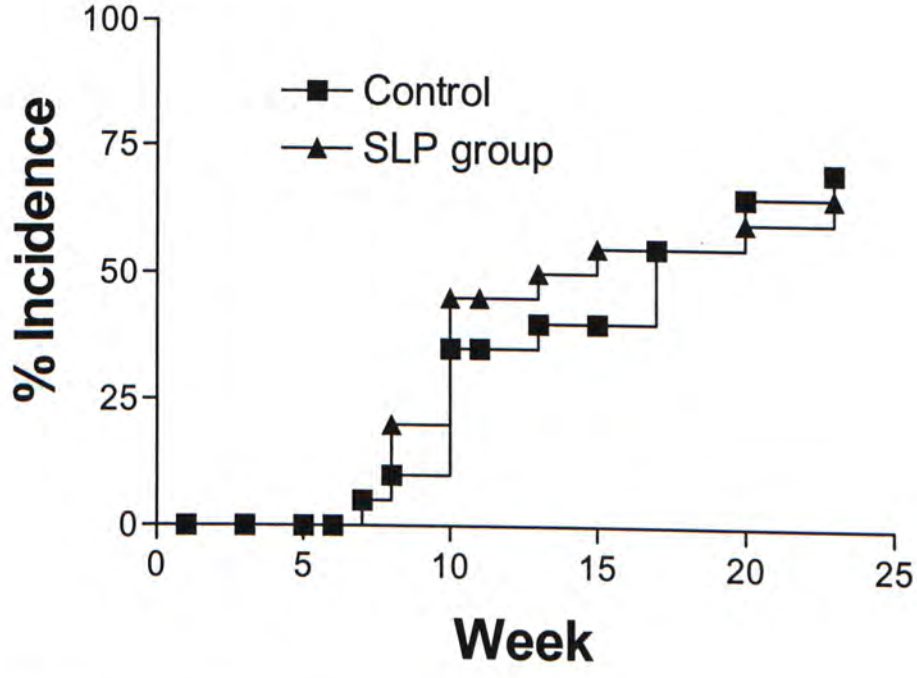


Figure 6.2 Effect of soy leaves supplementation on mammary tumor (n= 20 in each group). SLP: Soy leaves powder

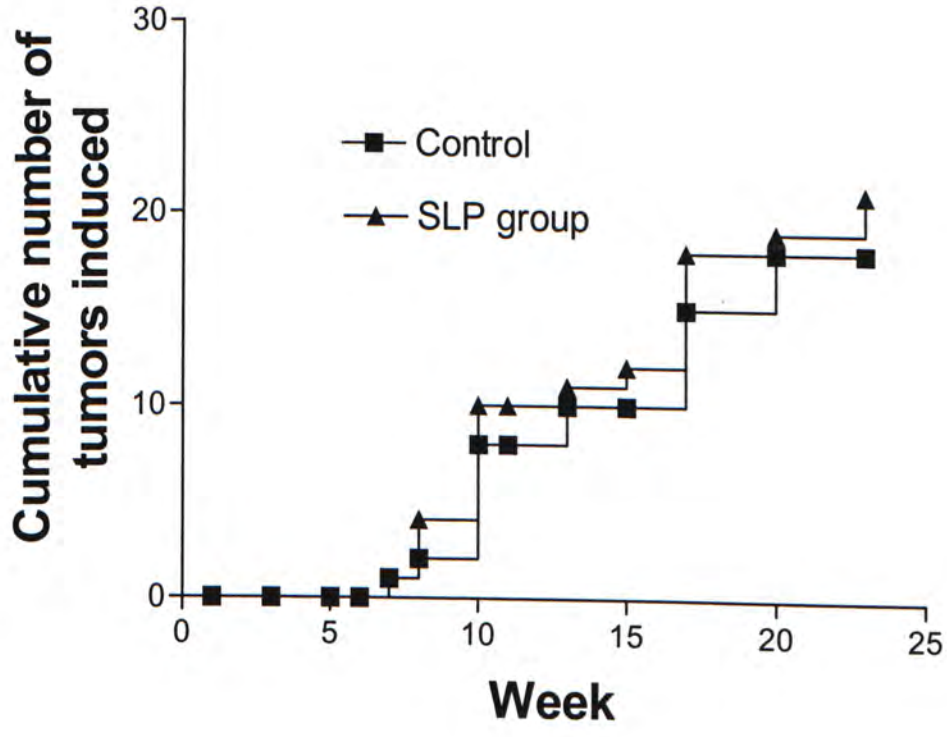


Figure 6.3 Effect of soy leaves on number of tumors induced (n= 20 in each group). SLP: Soy leaves powder

6.5 Discussion

Epidemiological data suggested that soybean may be beneficial in the prevention of certain cancers (Lee *et al.* 1991; Wu *et al.* 1998). It is believed that genistein and its glycosides are the active components in soybean which are responsible for this beneficial effect.

The growth of breast tumor cells is partly regulated *in vivo* through the action of estrogen. Estrogen may binds to and activates the cytosolic estrogen receptor (ER). Activated ER then translocates to the nucleus and initiates specific transcriptional events via its interaction with estrogen response elements on DNA. Estrogen can increase the levels of both growth factor receptors and their ligands.

Genistein, with a similar structure to estrogen, was proposed to have a weak estrogenic activity and can compete with estrogen for binding to the nuclear estrogen receptor (Martin *et al.* 1978). Setchell *et al.* (1984) suggest that genistein may inhibit tumor cell growth by an antiestrogenic mechanism through competition with E_2 for occupancy of the estrogen receptor (ER). Some studies review that genistein may also prevent tumor formation through the mechanisms of protein tyrosine kinase inhibition, topoisomerase II inhibition and inhibition of oxidation (Barnes and Peterson 1995b; Constantinou and Huberman 1995; Peterson 1995).

Although soy leaves contains genistein and its glycosides, their content were not

as high as that present in soybean (**Chapter 2, Table 2.4**).

The present study suggested that supplementation of 3% soy leaves powder in the diet could not decrease the incident rate of mammary tumor and the number of tumors induced in female Sprague-Dawley rats. This is probably because the amount of genistein and its glycosides present in soy leaves were not enough to inhibit tumor growth.

Chapter 7

Conclusions

Soybean (*Glycine max* L. Merr.) is one of the major food sources in China. It is usually consumed in the forms of soymilk and "tofu" (bean curd). Recently, many studies have been carried out to examine the health benefits of soybean. It was believed that isoflavone was the active component in soybean that is responsible for these health benefits. But there were no studies about the health benefits or chemical composition of soy leaves. In the present study, six different kaempferol glycosides were isolated from soy leaves. They are kaempferol-3-O-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→6)-β-D-galactopyranoside, kaempferol-3-O-(2,6-di-O-α-rhamnopyranosyl)-β-galactopyranoside, kaempferol-3-O-digalactopyranoside, kaempferol-3-O-diglucopyranoside, kaempferol-3-O-α-L-rharmnopyranosyl(1→6)-β-D-galactopyranoside and kaempferol-3-O-rutinoside. It was found that soy leaves also contained genistein, a kind of isoflavone, and its glycosides.

By using hamster as an animal model, supplementation of 3% soy leaves powder (SLP) or 3% soy leaves ethanol extract (SLEE) in their diet could significantly lower the ratios of non-HDL-C to HDL-C, an indicator in determining the risk of cardiovascualr diseases. The higher the ratio, the greater the chance of

getting cardiovascualr diseases. The excretion of neutral and acidic sterols were generally higher in these two groups. The decrease in the ratio of non-HDL-C to HDL-C in the SLP and SLEE groups implied that soy leaves supplementation in the diet could decrease the risk of cardiovascular diseases.

Soy leaves butanol extract showed weak antioxidative activity in Cu^{2+}-mediated oxidation of human low-density lipoprotein (LDL) *in vitro* while all the six kaempferol glycosides showed no antioxidative activity on human LDL. However, all the six kaempferol glycosides showed strong antioxidative activities on inhibition of erythrocytes haemolysis. The inhibition of erythrocytes haemolysis was in a dose-dependent manner. The present experimental results showed that the antioxidants in soy leaves may be able to decrease the oxidative stress. The present study suggest that the major antioxidant present in soy leaves which is responsible for the protection of LDL from Cu^{2+}-mediated oxidation has not been isolated. Further studies should be carried out to isolate unknown antioxidant(s).

In the blood vessel relaxing experiments, soy leaves butanol extract relaxed artery rings pre-constricted by U46619 or $PGF_{2\alpha}$ in a dose-dependent manner and this effect was independent of endothelium. The extract also inhibited the concentration-contraction curve to U46619 with slight reduction of the maximum contraction. The extract produced partial relaxation of phenylephrine (10 μM)-preconstricted both

endothelium-intact and –denuded rings. In contrast, the extract had no effect on the contractile response to 50 mM extracellular K^+. None of the six kaempferol glycosides affected vessel tension induced by U46619. A mixture of kaempferol glycosides prepared according to their relative composition in the extract had no effect either. Genistein induced a greater concentration-dependent relaxation than genistin. The present results indicated that soy leaves butanol extract caused endothelium-independent relaxation in rat carotid artery rings. The six kaempferol glycosides, accounting for ~48% the extract in weight, were not the ingredients responsible for the extract-induced relaxation. Genistein and genistin also caused relaxation, however, the dose range was beyond that of the extract causing relaxation. Further experiments are needed to identify the active components that actually account for the vasorelaxant effect of soy leaves extract and to examine their vascular action.

In the experiment of tumor induction, supplementation of 3% soy leaves powder in the diet could neither decrease the incidence rate nor the number of tumors induced. The present study indicated that soy leaves might not be able to prevent the development of mammary tumor or the amount of active components, genistein and its glycosides, in soy leaves were not enough to produce an inhibitory effect on tumor development.

In conclusion, soy leaves may possess some beneficial effects in humans

through the actions of modifying the lipoprotein profile to lower risk of cardiovascular disease, scavenging of the free radicals to decrease the oxidative stress and dilating the arteries to decrease the risks caused by hypertension.

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